Cloning and localisation of nematode inhibitory amino acid receptors

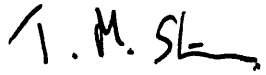
Skinner, Thomas Morland

Award date:
1997

Awarding institution:
University of Bath

CLONING AND LOCALISATION OF NEMATODE INHIBITORY AMINO ACID RECEPTORS

Submitted by Thomas Morland Skinner
for the degree of PhD
at the University of Bath
1997



Thomas M. Skinner

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Acknowledgements

I would like to thank my supervisors, Adrian Wolstenholme and George Lunt, for their advice and help throughout my time in Bath.

Merck Sharp and Dohme generously provided CASE sponsorship for this research.

The present members of lab 0.47, and past members of 3.44, have been exceptionally helpful and supportive when things went wrong, and keen to celebrate when things worked out. I couldn't have hoped for a happier lab. in which to cut my teeth.

I am grateful to Gerald Coles and Anita at Lower Langford who have kept us in worms and eggs when all other supply lines failed.

I thank the many people who have given freely of their time and experience, both in answering questions and giving technical assistance. In particular, Lindy Holden-Dye, Zainab Bascal and David Brownlee for help with IC, Bill Bennet for his publishing skills. David Tosh for help with the confocal, David Laughton for help with everything, and anyone who came in on monday morning to find I had annexed their bench space.

Thanks most of all to Cecily, who has put up with my rantings over the past year.

Abstract

An antiserum was raised against HGP1, a synthetic peptide epitope corresponding to amino acid residues 46-61 from the N-terminal extracellular domain of the putative inhibitory amino acid receptor subunit Hg1 (EMBL accession number X73584). ELISA analysis confirmed that anti-HGP1 bound to HGP1 with a half maximal response at 1:700 dilution. A recombinant form of the extracellular domain of Hg1 was produced by expression as a fusion protein in *Eschericia coli*. The antiserum also bound specifically to this protein on Western blots of transformed cell lysates, at a dilution of 1:1000.

Immunocytochemistry was used to localise HGP1 immunoreactivity to the post-synaptic membranes of the neuromuscular junction in *Haemonchus contortus* and *Ascaris suum*. 3-5 neurons in the nerve ring of *H. contortus*, similar to the *Caenorhabditis elegans* RM (ring motorneuron) and RI (ring interneuron) classes, and a single unidentified cell with its cell body in the region of the lateral ganglion were also immunoreactive. This distribution is consistent with Hg1 acting as a subunit of a GABA receptor *in vivo*.

A partial cDNA clone encoding part of two putative glutamate receptors, Hg2 and Hg3, has already been described (Laughton 1993). The Polymerase Chain Reaction (PCR) was used to clone the remaining 5' end of this cDNA. The Hg2/3 mRNA undergoes alternative splicing to yield mature mRNAs encoding Hg2 and Hg3, which share 79% and 87% identity with their probable *C. elegans* orthologues Cegbr2 and Cegbr3, and may represent members of a new subclass of inhibitory glutamate receptor subunits, GluClγ.

Contents

Figures

Tables

Abbreviations

ACh	Acetylcholine
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
dNTP	Deoxyribonucleoside Triphosphate
EDTA	Ethylenediaminetetracetic Acid
EST	Expressed Sequence Tag
FaRP	FMRFamide Related Peptide
GABA	γ-Aminobutyric Acid
GI	Gastrointestinal
5-HT	5-Hydroxytryptamine (Serotonin)
IC	Immunocytochemistry
IGluR	Inhibitory Glutamate Receptor
IPTG	Isopropylthio-β-D-Galactoside
MBS	Maleimidobenzoic acid Succinamide ester
MMLVRT	Murine Maloney Leukemia Virus Reverse Transcriptase
mRNA	Messenger RNA
nAChR	Nicotinic Acetylcholine Receptor
NMJ	Neuromuscular Junction
OAc	Acetate Anion
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Rection
PEG	Polyethylene Glycol
PFA	Paraformaldehyde
RACE	Rapid Ampification of cDNA Ends
RNA	Ribonucleic Acid
RNase	Ribonuclease
rNTP	Ribonucleoside Triphosphate
SDS	Sodium Dodecyl Sulphate
Taq	*Thermus aquaticus*
TBPS	t-Butylbicyclo-phoshorothionte

TGIC	Transmitter Gated Ion Channel
TM	Transmembrane domain

1. Introduction

1.1 The phylum nematoda

Over twenty thousand species members of this phylum have been described from a wide range of environments including the sea, freshwater systems, the soil and as parasites of plants and animals. This diverse range of habitats is reflected in a diverse range of lifecycles, however despite these differences some characteristics are common to all nematodes. Larval forms of all species undergo four moults, passing through four larval stages (L1-L4), before reaching maturity. Furthermore the gross anatomy of all species also shows marked similarities. All nematodes share a common basic layout, being comprised of two concentric tubes, the body wall and gut, separated by the pseudocoelom, a fluid-filled space. The body is unsegmented having only longitudinal muscles, thus movement occurs by the propagation of a coordinated sinusoidal wave of muscular contraction which acts against internal hydrostatic pressure. The nervous systems of all members of the phylum contain a similar number of neurons with a well conserved connectivity; individual neurons described in *Caenorhabditis elegans,* a small (2mm) microbivorous free-living nematode, have been shown to have counterparts in *Ascaris suum,* a larger (30cm) parasitic species (Angstadt *et al.* 1989, Johnson and Stretton 1985, Holden-Dye and Walker 1994). The anatomy of *C. elegans* is shown in Figure 1.

The largest organ is the gut, which extends posteriorly from the pharynx at the anterior end of the worm for most of the length of the body to the anus, which is located ventrally. The pharynx is a muscular pumping organ which has two functions; the ingestion and physical disruption of food and the maintenance of internal hydrostatic pressure. The varied feeding strategies of nematodes have led to the evolution of marked variations in pharynx structure; whilst *C. elegans* has a large pharynx with two pronounced swellings, the pseudo-bulb and posterior bulb, haematophagous species of the order strongylida have relatively undifferentiated pharynxes (Bird 1971). Most nematode species are free-living, however the many parasitic species cause medically and economically important diseases of humans and animals (Politz and Philipp 1992).

1.1.1 Nematodes and disease

1.1.1.1 Nematodes of veterinary importance

The species of greatest veterinary importance in Britain and N. Europe are *Ostertagia ostertagi, O. circumcincta* and *Haemonchus contortus*, all members of the order *Strongylida,* and *A. suum*

order *Ascaridida* (G. Coles, Personal Communication.). All are gastrointestinal (GI) nematodes; *A. suum* infects pigs, *O. ostertagia* is parasitic on cattle, whereas the other two affect sheep and goats. The life cycle of *H. contortus* is typical of the strongyles.

1.1.1.1.1 *Haemonchus contortus*

H. contortus is a haematophagous nematode which parasitises the abomasum of a range of hosts including sheep, goats, llamas and alpacas. The adult worms reach 2-3cms in length when mature (Figure 2) and are found attached to the wall of the abomasum where they feed on host blood. The species is dioecious, the males having the well-developed bursa that is characteristic of the order. Mating occurs in the abomasum from where fertile eggs are released into the faeces. The early larval phases are microbivorous and develop in the dung, hatching 2-3 days after defecation and reaching the infective L3 stage in approximately 2-3 weeks. The L3s exhibit marked geotrophism and climb up grass stems where they are ingested by a grazing host. The next two moults occur after the infective larvae have reached the abomasum and burrowed into the gut wall.

Each adult worm consumes about 15µl of host blood per day, representing a significant burden to an infected host which may be carrying several thousand worms. This blood loss can cause anaemia, loss of condition and even death in heavily infected or otherwise weakened individuals (Smyth 1994), representing a significant cost to the sheep farming industry. In Australia alone the annual cost in lost production and anthelminthic expenditure has been estimated as Aus$500 million (Emery *et al.* 1993), the true global price of damage wrought by nematode parasites of livestock is likely to be much higher.

Figure 1. Adult male C. elegans.

Legend. (*b.c.*) buccal cavity, (*p.*) pharynx, (*n.r.*) nerve ring, (*ex.*) excretory system, (*g.*) gut, (*t.*) testis, (*s.*) spicules, (*b.*) bursa. Scale bar is approximate.

Adapted from Smyth 1994.

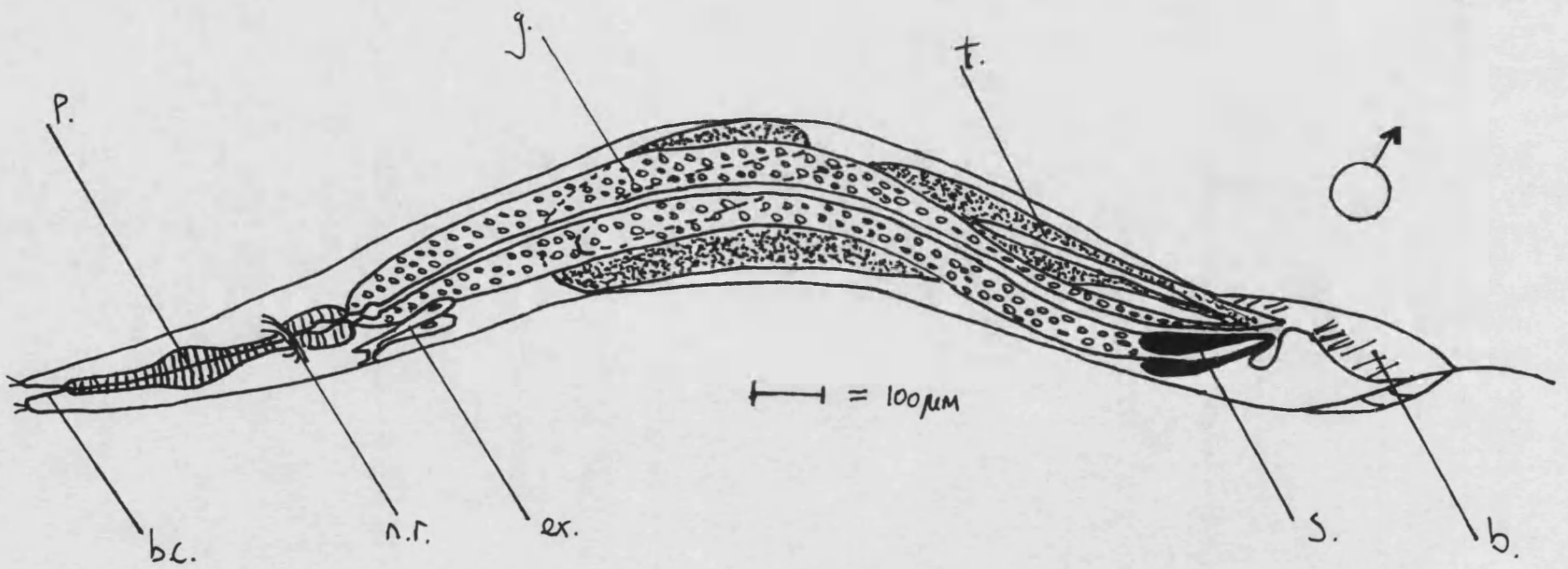


Figure 2. Adult H. contortus.

Scale: approximately 5x life size

1.1.1.1.2 *Ascaris suum*

A. suum is closely related to the human parasite *A. lumbricoides*, which affects over 200 million people worldwide (WHO 1996). There is some disagreement over the taxonomy of *A. lumbricoides* and *A. suum*; some authors consider *A. lumbricoides* a discrete species whereas others have postulated that *A. lumbricoides* is a sub-species of *A. suum* and should therefore be called *A. suum* var. *lumbricoides* (Nadler 1987). The former nomenclature is adopted in this thesis for simplicity.

A. suum infects pigs following the ingestion of viable eggs, which contain developed but unhatched L2s. The larvae hatch in the duodenum and burrow into the mucosa, from where they migrate to the liver. The L2s undergo a moult here before entering the bloodstream which carries them to the capillaries of the lungs. Approximately a week after ingestion, the L3s move into the alveoli and up the trachea, where they re-enter the digestive system. The final moults occur in the intestine and the free-swimming adults are fully mature in approximately 60 days (Smyth 1994, Crompton *et al.* 1989).

1.1.1.2 Nematodes of importance to human health

1.1.1.2.1 GI nematodes

Several species of nematodes parasitise the alimentary tract of man. *Enterobius vermicularis* and the whipworm *Trichuris trichiura* are found only in the intestine whereas *Ascaris lumbricoides* and the hookworms *Necator americanus* and *Ancylostoma duodenale* also parasitise other organs during their development. Infection with any of these species, with the exception of *E. vermicularis*, can cause intestinal bleeding and blockage. Approximately 200 million people are estimated to be infected with *A. lumbricoides*, which although only a direct cause of death to weakened or exceptionally heavily infected hosts, is nevertheless a significant cause of morbidity (Brooks 1963, WHO 1996).

1.1.1.2.2 Filarial nematodes - *Onchocerca volvula, Brugia malayi* and *Wucheria bancrofti.*

Onchocerciasis affects 18 million people, with another 120 million at risk. Infective microfilariae are transmitted to man during a bite from an infected intermediate host, the blackfly *Simulium damnosum*. Development of the worms continues sub-cutaneously causing debilitating skin disease and blindness (WHO 1996). *Brugia malayi* and *Wucheria bancrofti* rely on the mosquito genera *Aedes, Mansonia, Culex* and *Anopheles* as intermediate hosts. Infection occurs

in a similar way, however L3 larvae migrate to the lymph nodes where they mature causing blockage of lymphatic flow and concomittant inflammation. The resulting lymphatic filariasis is usually confined to the groin, genitals and lower limbs (Anderson 1992). Adult females release ensheathed microfilariae into the blood by where they circulate with a marked periodicity, synchronous with the feeding habits of their preferred intermediate host species. When ingested as part of a blood feed by a biting mosquito, they exsheath and continue development to the infective L3 stage. Filariasis is the cause of morbidity in approximately 120 million people worldwide (WHO 1996).

1.1.1.3 Control methods

The huge medical and economic cost of the damage inflicted by nematode pathogens has been ameliorated by a range of control strategies. Effective long term control may be simple or require a combination of approaches, depending on the lifecycle and infective route of the parasite. Prophylactic strategies have been devised for some species; these entail physically preventing infection, destroying intermediate hosts or reservoir populations, or immunisation. Chemotherapeutic intervention is used both prophylactically and as a symptomatic treatment post-infection and is the predominant control method for both veterinary and medical infections.

1.1.1.3.1 Chemotherapeutics

Four main classes of nematocidal drugs are in general medical and veterinary use (Figure 3). They differ both in their sites of action and effective species range. As resistance to all four drug classes has been demonstrated in the field, there is an urgent need both for the development of new drugs and for basic research into nematode biology and resistance mechanisms (Coles *et al.* 1993).

The Benzimidazoles.

The benzimidazoles exert their effect by binding to β-tubulin and thereby inhibiting microtubule assembly. Although β-tubulins are highly conserved proteins which are found in both host and parasite, the benzimidazoles show comparatively low toxicity to mammals. Their selectivity derives from the much higher rate of dissociation of benzimidazoles from mammalian tubulins than from parasitic orthologues (Lacey and Gill 1994). Unfortunately the intense and often inappropriate use of these antimitotic drugs in agriculture has led to the widespread development of resistance in nematode populations (Coles *et al.* 1991) . Resistance is associated with a point mutation in the β_1-tubulin gene; a single nucleotide change causes an amino acid change in the β_1

protein from phenylalanine (Phe) to tyrosine (Tyr) at residue 200. This highly conservative substitution, resulting in the net gain of one hydroxyl group, is sufficient to confer benzimidazole resistance (Kwa *et al.* 1994 & 1995). Benzimidazoles were originally used as anti-fungal agents, the development of resistance in fungi is also caused by a point mutation in a similar region of the β_1-tubulin protein, glutamic $acid_{198}$ to lysine or glycine (Wheeler *et al.* 1995).

Piperazine

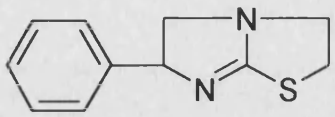
Piperazine causes a Cl^- dependent flaccid paralysis and concommitant membrane hyperpolarisation when applied to nematode body wall muscle preparations (Martin 1982). Both γ-aminobutyric acid (GABA) and piperazine act on muscle cells at chloride channels with similar biophysics, with a conductance of 22 pS (Martin 1987) suggesting that piperazine acts as an agonist at a neuromuscular GABA receptor. Piperazine is used medically against free-swimming intestinal worms such as *E. vermicularis* and *A. suum*, resulting in their expulsion by normal bowel movement.

Levamisole

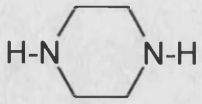
Levamisole induces a tetanic muscular paralysis in a wide range of nematodes and appears to mimic the effect of acetylcholine (ACh) (Reviewed in Geary *et al.* 1992). Mutant *Caenorhabditis elegans* strains which do not express a full complement of ACh receptor subunits are insensitive to levamisole and levamisole binding to wild-type worms is inhibited by ACh, suggesting that levamisole acts as a competitive agonist at the nematode acetylcholine receptor (Lewis *et al.* 1987). This hypothesis has been proved by patch clamp electrophysiology on muscle cells; at the single channel level both levamisole and ACh open cation selective channels with similar mean open times within similar concentration ranges (1-90 & 1-100 μM respectively). Levamisole is a large cation in aqueous solution and causes channel block at concentrations over 30μM (Martin 1993).

Figure 3. The chemical structures of the four main anthelminthic classes

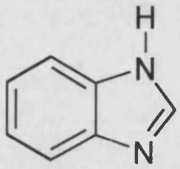
Levamisole



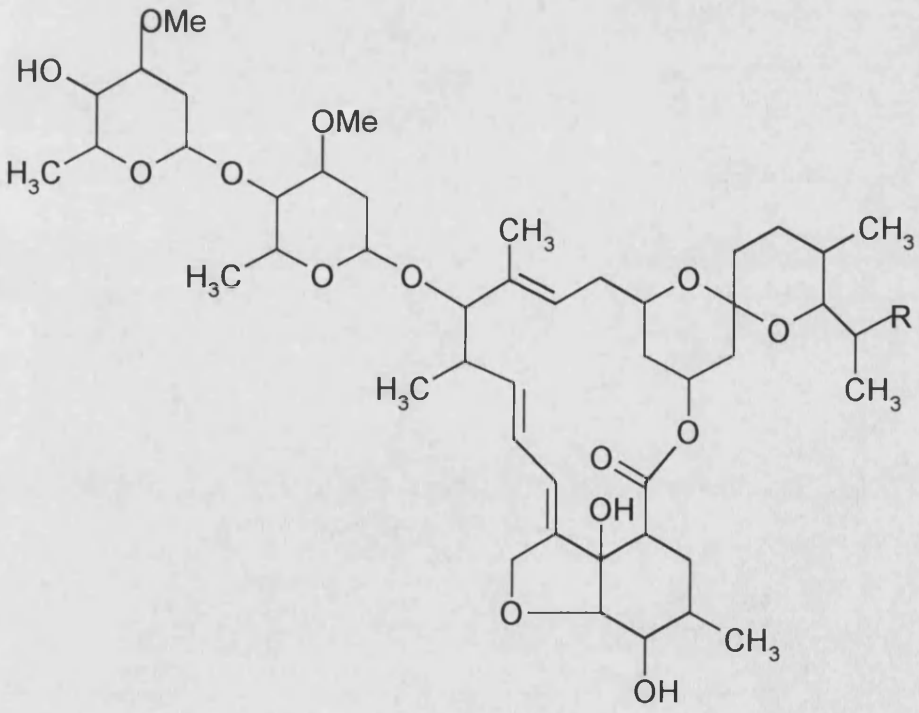
Piperazine



Benzimidazole



Ivermectin



Avermectins and milbemycins

Ivermectin, milbemycins and their structural analogues are members of a family of macrocyclic lactones which exert a potent anthelminthic effect at nanomolar concentrations. Preliminary data showed an irreversible stepwise opening of chloride channels on *A. suum* somatic muscular at micromolar concentrations (Holden-Dye *et al.* 1989, Holden-Dye and Walker 1990), however the avermectins have also been shown to act at a glutamate gated chloride channel in *Xenopus oocytes* transfected with *C.elegans* mRNA, with a ranking of activity and affinity which correlates well with their nematocidal potency (Arena 1995). An avermectin preparation for medical use, "mectizan", has been administered by the WHO to those suffering from or at risk of onchocerciasis in Africa and Central America. Annual treatment with mectizan has been effective in controlling the symptoms of the disease, although at the dosage used the anthelminthic is ineffective against adult worms.

1.1.1.3.2 Vaccination

Parasitic nematodes often induce a strong immune response in their hosts which can have a profound effect on the level of infection (Jarrett *et al.* 1968). Sheep presented with a chronic challenge of infective L3 *H. contortus* from contaminated pasture exhibit a marked increase in worm burden (the periparturient rise) during pregnancy, which is associated with suppression of the hosts immune system. In contrast, prolonged infection may lead to "self-cure" (Smyth 1994, Jarrett *et al.* 1968) in which the host organism mounts a strong and successful immune response to the infection. From this evidence it is apparent that the level of *H.contortus* infection is strongly influenced by the host immune response, therefore the artificial stimulation of a protective immune response by vaccination is an attractive goal for the control of this species. Host immune response during infection are raised predominantly against excretory/secretory (ES) products and cuticular epitopes, however promising vaccines are now in development which have been derived from hidden antigens, such as gut membrane proteins, purified from haematophagous nematodes (Andrews *et al.* 1994, Munn *et al.* 1993). These antigens are usually hidden from the host's immune system as white blood cells ingested by the nematode are destroyed by digestive processes and consequently no immune response is mounted. Several such vaccines are now in veterinary trials and, for blood-feeding strongyles at least, this strategy shows much promise (Emery *et al.* 1993)

1.1.1.3.3 Biological control

The nematophagous fungi *Monacrosporium eudermatum, Arthrobotrys oligospora* and *A. robusta* have been used successfully to reduce levels of infective L3 *H. contortus* in faecal culture (Mendoza-De Gives and Vazquez-Prats 1994). The agricultural value of this control strategy has yet to be determined as it requires either the application of large numbers of fungal conidia directly onto pasture, or the production of an oral dose formulated to release viable spores after passage through the hosts gastrointestinal tract. The efficacy of this approach is also affected by climatic conditions. Bacterial, viral and protozoan diseases of nematodes have received little study, but may offer potential for the control of nematode parasites of livestock (Waller 1992).

1.1.1.3.4 Other control methods

Some nematode diseases can be effectively cured by simple changes of habit and hygiene. *E. vermicularis* infection can be prevented by a strict hygiene regime involving bathing before meals and immediately upon rising; indeed most nematode parasites of man which infect by an oral route can be controlled effectively by improved sanitation, drinking water and hygiene (Jarry 1967). Strongyle infection of livestock can be reduced by frequent pasture rotation, which removes animals from possible infection by the L3 stage. This technique is expensive however, as the resulting stock density is much lower than that attainable using conventional grazing strategies. In addition, there is some evidence to suggest that pasture rotation alone is an ineffective control strategy (Taylor *et al.* 1991).

1.2 Nematode neurobiology

Unfortunately, resistance to all four classes of anthelminthics discussed above has been recorded in the field and the most potent family of nematocides, the avermectins, are excreted in the dung where they persist with toxic effects on colonising insects (Strong *et al.* 1996). As dung colonising insects represent an important food source for a host of insectivorous grassland species the full ecological effect of the avermectins is still unknown. The development of drug resistance and increasingly strict environmental legislation underlines the urgent need for the development of new drugs. As the majority of the anthelminthic classes currently used in human and veterinary medicine act at receptors within the nematode nervous system, its continued study should yield novel targets for use in mechanism-based screening programmes (Geary *et al.* 1992).

The neuroanatomy and neuropharmacology of the phylum are important areas of research for several other reasons. This simple, defined anatomy permits the localisation of receptors and neurotransmitters to individual neurons with a precision that can not be matched in any other organism. This data can be used in conjunction with characterised mutants and laser ablation experiments to define the individual neurons, neurotransmitters and receptors involved in nematode behaviour patterns (reviewed in Thomas 1994).

1.2.1 The anatomy of the nervous system and neuromusculature

The nervous system of the hermaphrodite *C. elegans* consists of 302 neurons (White *et al.* 1986). Twenty of these are found in the pharyngeal nervous system, which is connected to the rest of the nervous system by two neurons. The remaining neuropile is concentrated in the circumpharyngeal nerve ring and associated ganglia. Nerve tracts project from this structure in both anterior and posterior directions. Anterior tracts carry the processes of predominantly sensory neurons from the cephalic sensilla, whereas the lateral, dorsal and ventral nerve cords contain the posterior proceses which are connected at regular intervals by lateral commissures. The posterior tracts include the body muscle motorneurons and connect to the four posterior ganglia in the tail. Figure 4 shows the major nerve tracts in the head of *A. suum.*

Nematode motorneurons do not have dendritic presynaptic processes, instead the muscle cells send out narrow arms to synapse with dorsal and ventral nerve cords (Stretton 1976). This unusual form of neuromuscular anatomy (Figure 5) is not unique to nematodes and is found in the starfish *Astropecten irregularis* and other marine invertebrates. (Walker *et al.* 1993).

Figure 4. The cephalic nervous system of the adult A.lumbricoides

Legend. (*d.g.*) dorsal ganglion, (*l.g.*) lateral ganglion, (*ex.g.*) extremolateral ganglia, (*v.n.*) ventral nerve cord, (*l.n.*) lateral nerve cord, (*d.n.*) dorsal nerve cord, (*n.r.*) nerve ring, (*a.g.*) amphidial ganglia, (*v.g.*) ventral ganglion, (*lv.n.*) lateroventral nerve cord, (*rv.g.*) retrovesicular ganglion.

Adapted from Smyth 1994.

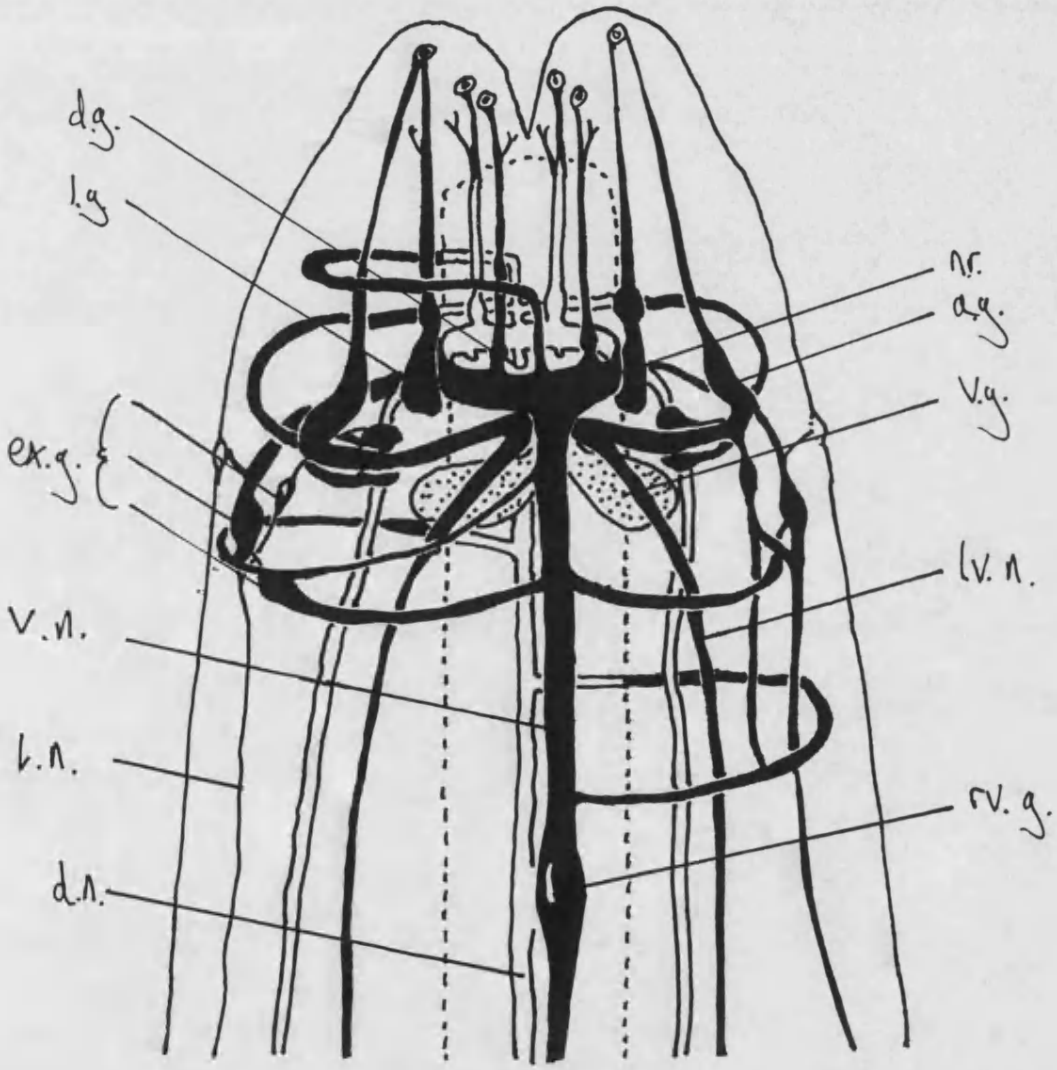
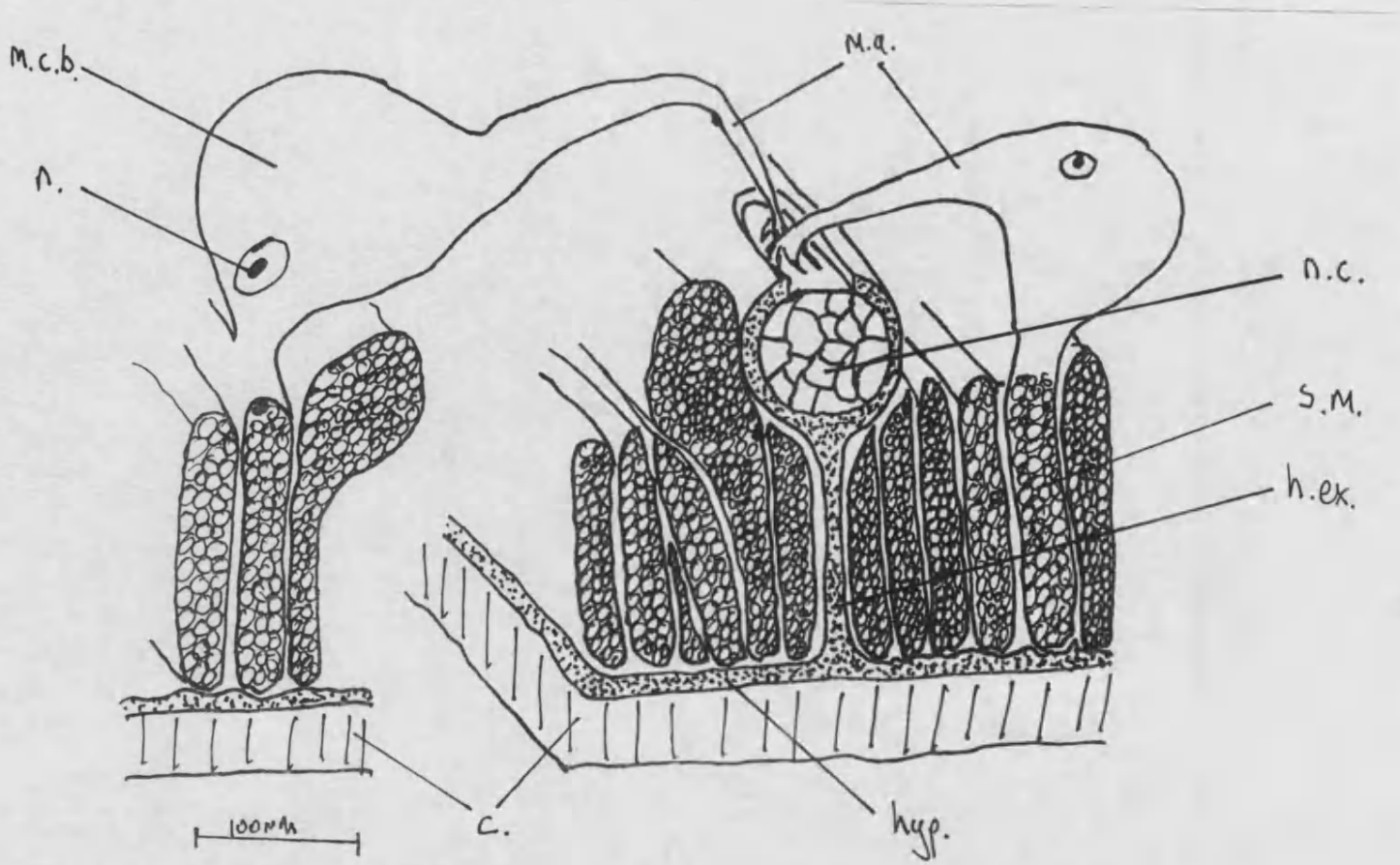


Figure 5. The nematode neuromuscular junction.

Legend. (*m.c.b.*) muscle cell body or "bag", (*n.*) nucleus, (*m.a.*) muscle arms, (*n.c.*) nerve cord, *(s.m.*) striated muscle, (*h.ex.*) hypodermis extension, (*hyp.*) hypodermis, (*c.*) cuticle. Scale bar approximate.



1.2.2 Model nematodes for neurobiology

1.2.2.1 Caenorhabditis elegans - genetics

The free-living nematode *C. elegans* is well suited to genetic experiments as it can be bred through a sexual cycle using male/hermaphrodite crosses, or by selfing of the hermaphrodite. An additional advantage is that the organism has a short lifespan and can be cultured easily in the laboratory. Transformation and transposon mediated gene knockout techniques are well developed (Fire 1986, Fire *et al.* 1990, Anderson 1995), so the effect of up- and down-regulation of individual genes can be studied. The lineage of every somatic cell is known and the complete connectivity of the nervous sytem of the adult hermaphrodite has been mapped (White *et al.*1986). To date approximately 80% of its genome has been sequenced.

1.2.2.2 Ascaris suum - electrophysiology and immunocytochemistry

Its large size, relative abundance in the UK and similarity to the important human parasite *A. lumbricoides* make *A. suum* an ideal nematode for study. The neuroanatomy of this nematode has been studied in detail (Angstadt *et al.* 1989, Holden-Dye and Walker 1994) and found to differ surprisingly little from the *C. elegans* system. This similarity has led to the suggestion that *A.suum* can be used as a large-scale model of the *C. elegans* nervous system (Martin 1993, Holden-Dye and Walker 1994) as *A. suum*'s large muscle cells, neurons and pharynx, unlike those of *C. elegans*, are amenable to electrophysiology.

1.2.3 Nematode neurotransmitters

1.2.3.1 Classical neurotransmitters

Our understanding of the distribution of classical transmitters in nematodes derives partly from direct studies, using immunocytochemical and histological techniques, and partly from the pharmacology of nematode tissues and distribution of synthetic enzymes and membrane transporters. The roles played by individual neurotransmitters in specific nematode behaviour patterns have been defined by direct pharmacological investigation, the study of *C. elegans* genetic mutants and laser ablation of specific neurons (McIntire *et al.* 1993a,b, Thomas 1994).

1.2.3.1.1 GABA

GABA was first shown to depolarise *A. suum* muscle cells by Del Castillo (1964), subsequent work has confirmed its role as the major inhibitory transmitter at the nematode neuromuscular junction. Consistent with this, GABA deficient *C. elegans* mutants exhibit uncordinated (*unc*)

phenotypes and GABA-immunoreactivity has been demonstrated in the inhibitory motorneurons of *A. suum* (Johnson and Stretton 1985, McIntire *et al.* 1993b). GABA receptors are found extrasynaptically on muscle cell bodies of this species and although the physiological significance of these receptors is not clear, their pharmacologies have been studied using whole-cell and patch-clamp electrophysiological techniques (Martin 1980, 1982, Holden-Dye *et al.* 1989 Holden-Dye and Walker 1988). The extrasynaptic muscle GABA receptor has biophysical properties and agonist pharmacology broadly similar to those of the vertebrate $GABA_A$ receptor; muscimol and S-(+)-dihydromuscimol gate the channel with potencies of 0.40 ± 0.02 & 7.53 ± 0.98 respectively relative to GABA (Holden-Dye *et al.* 1989, Martin 1993). Conversely nematode GABA receptors exhibit an unusual antagonist profile and are insensitive to the channel blocker picrotoxin, competitive antagonist bicuculline and the modulatory benzodiazepines (Martin 1993, Holden-Dye *et al.* 1989, Walker *et al.* 1993). The $GABA_B$ agonist baclofen has no effect (Holden-Dye and Walker 1988).

In view of the important inhibitory role played by GABA in the nematode neuromuscular system, it is unsurprising that most of the GABAergic neurons identified in the nematode species studied to date are motorneurons. A set of 26 GABAergic neurons has been defined for *C. elegans*. All but one of these are motor neurons; six DD, 13 VD, 4 RME 1DVB and 1 AVL motorneuron are stained by an antiserum raised against GABA (McIntire *et al.* 1993b). The DD and VD neurons are necessary for coordination of the sinusoidal body wave involved in locomotion. *unc*-25 GABA synthesis deficient mutants and wild-type (N2) individuals with laser ablated DD and VD neurons exhibit a simultaenous twitch of both ventral and dorsal muscles in place of the usual wave of contraction. RME motorneurons control the extent of head movement , *unc*-25 and RME ablated individuals exhibit a "loopy" phenotype, characterised by an excessive head deflection during feeding.

The AVL and DVB neurons are unusual, being both excitatory and GABAergic . Both cells are necessary for efficient defaecation and appear to mediate their effect via an excitatory GABA receptor (*ibid.* and Reiner and Thomas 1995). The RIS interneuron has an unknown function and RIS ablated individuals display a wild-type phenotype.

The distribution of GABA-immunoreactivity in *A.suum* is very similar to that seen in *C. elegans*, concentrated at motorneurons (Johnson and Stretton 1987). No staining could be ascribed to a cell corresponding to the *C. elegans* RIS neuron , although one cell in the rectal ganglia and two pairs of amphid and deirid neurons in the lateral ganglia stained (Guastella *et al.* 1991, Guastella and Stretton 1991). Two amphid neurons also displayed GABA-like immunoreactivity in

Goodeyus ulmi (Leach *et al.* 1987), suggesting that in addition to its ubiquitous role at the somatic and enteric musculature, GABA has a role in the processing of sensory information in some species.

1.2.3.1.2 Acetylcholine

There is a wealth of published data which proves that ACh functions as the major excitatory neurotransmitter throughout the phylum. Early work on *A. suum* demonstrated that ACh and nicotine both contract *A. suum* muscle strips and cause membrane depolarisation of individual muscle cells (Baldwin and Moyle 1949, Del Castillo *et al.* 1963, Segerberg and Stretton 1993). Identified excitatory motorneurons (DE1-3) in *A. suum* have been shown to contain high levels of choline acetyltransferase (CAT) activity, whereas known inhibitory neurons contained CAT activity which was not significantly different from controls (Johnson and Stretton 1985). The neuromuscular ACh receptor has been extensively studied using whole-cell and patch-clamp electrophysiology on *A. suum* muscle preparations and shown to be broadly similar to the vertebrate nicotinic acetylcholine receptor (nAChR): ACh induces depolarisation and an increase in input conductance of the somatic muscle cells of *A. suum* with an EC_{50} of 10 ± 2 μM, this response is reduced by two orders of magnitude upon application of 100μM d-tubocurarine, a nAChR antagonist (Colquhoun *et al.* 1991, 1992, Segerberg and Stretton 1993). Similar experiments with the muscarinic antagonist N-methyl scopolamine showed only 5-fold reduction at the same antagonist concentration. (Segerberg and Stretton 1993). Comparison of the agonist profiles of vertebrate and nematode AChRs showed that whilst the nemtatode AChR is nicotinic in nature, it shows considerable pharmacological differences from both ganglionic and neuromuscular isoforms of the vertebrate nAChR (Colquhon *et al.* 1991). In addition to its involvement in locomotion, acetylcholine is also implicated in egg-laying in *C. elegans*, cholinergic agonists induce egg-laying in wild type strains and rescue some egg-laying defective mutant phenotypes (Trent *et al.* 1983, Weinshenker *et al.* 1995).

1.2.3.1.3 5-HT

C. elegans deficient in 5-HT show abnormal phenotypes for male mating and this neurotransmitter is involved in egg laying (Loer and Kenyon 1993). Intriguingly, 5-HT deficient mutants exhibit normal egg-laying phenotypes, suggesting that this neurotransmitter acts in conjunction with other neurotransmitter systems (Weinshenker *et al.* 1995). *A. suum* pharynx preparations are stimulated from a resting quiescent state to active pumping (0.5hz) upon application of 100μm 5-HT (Brownlee *et al.* 1995). Injected 5-HT has a complex inhibitory effect on locomotion and induces a profound ventral tail curl (Reinitz and Stretton 1996).

1.2.3.1.4 Glutamate

The distribution of this neurotransmitter is poorly understood in nematodes. The ubiquity of glutamate as an amino acid makes it difficult to differentiate between neurotransmitter and free intracellular glutamate. As a result of this, the distribution of glutamate in the nervous system has been inferred from the pharmacology of nematode tissues and the distribution of glutamate transporters and receptors.

Several independent experiments suggest that the M3 motorneurons, which innervate the pharynx, are glutamatergic. Pharyngeal pumping is inhibited by applied glutamate (D. Brownlee, personal communication) and the glutamate receptor subunits GluClβ and GluClα_2 are expressed in the pharynx (Laughton *et al.* 1997, J. Dent, personal communication). Analysis of deletion mutants of *glr*-1, which shares 40% identity with vertebrate α-amino-3-hydroxy-5-methyl-isoxazole (AMPA) type ionotropic glutamate receptors, suggested that the ASH sensory neurons are also glutamatergic (Maricq *et al.* 1995, Hart *et al.* 1995). Reporter gene analysis of the expression pattern of *glr*-1 showed that at least 17 neurons express *glr-1*, from which the authors inferred the existence of other glutamatergic neurons. Furthermore, work by Radice and Lustigman (1996) showed that a presumptive glutamate transporter is expressed in 14 neurons, the anterior hypodermal cells and the pharynx.

1.2.3.2 Neuropeptides

1.2.3.2.1 FMRFamide related peptides - FaRPs

The cardioexcitatory peptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) was first isolated from the clam *Macrocallista nimbosa* and shown to have an excitatory effect on molluscan heart and radula preparations with a threshold concentration of ~10^{-8}M (Price and Greenberg 1977). Atkinson *et al.* (1988) used immunocytochemistry with antisera raised against synthetic FaRPs to demonstrate the existence of this neuropeptide system in nematodes. The isolation of a nematode FaRP denoted AF1 was reported from Stretton's laboratory soon after (Cowden *et al.* 1989), HPLC separation of acid-methanol *A. suum* extracts led to the purification of 11 other FaRPs from this species (Cowden and Stretton 1993,1995). Further FaRP sequences have been implied from the sequencing of FaRP genes (Genbank acc. no. U15279). In total, over 22 FaRP sequences have been published from *A. suum, C. elegans, H. contortus* and *P.redivivus* (Brownlee *et al.* 1996 and refs therein).

AF2 (KHEYLRFamide) is found in all four species (Cowden and Stretton 1993, Marks *et al.* 1995, Maule *et al.* 1994 and Keating *et al.* 1995). Application of AF1 and AF2 to *A. suum* muscle strip preparations resulted in similar biphasic responses composed of an initial pre-synaptically mediated inhibitory effect, followed by a prolonged phase of rythymic contraction mediated by post-synaptic receptors . Interestingly AF2 is myoactive at much lower concentrations than AF1; AF2 has a threshold concentration of 10pM compared to 1 nM for AF1 (reviewed in Maule *et al.* 1996). There is compelling evidence that AF2 is of major physiological importance. Firstly, as described it has exceptional potency at *A. suum* somatic musculature (Cowden and Stretton 1993), showing threshold concentrations orders of magnitude lower than any other FaRP studied. Secondly, AF2 is found in relative abundance in the four nematodes studied in any depth. Thirdly, indirect immunocytochemistry using monoclonal antibodies and AF2-adsorbed anti-FMRFamide has shown AF2 staining to be a major component of total FMRFamide immunoreactivity. (Davies and Stretton 1997, Keating *et al.* 1995)

PF4 induces a chloride dependent relaxation of *A. suum* muscle preparations, but does not act at the same receptor as GABA (Martin *et al.* 1995 in Maule *et al.* 1996). Other FaRPs assayed have shown excitatory, inhibitory and biphasic effects on muscle strips. A full discussion of this data is beyond the scope of this thesis and can be found in the neighbouring reviews of Brownlee *et al.* (1996) and Maule *et al.* (1996).

The discovery of such a complex neuropeptide system has put paid to the notion that the simplicity of nematode neuroanatomy might be mirrored by a simple neurochemistry. Despite the large body of work on nematode neuropeptides already published the effect of FaRPs on individual neurons has not been studied in depth and the cloning of a FaRP receptor has yet to be reported from the phylum. FaRP specific monoclonal antibodies should permit the accurate localisation of individual FaRP species (Davies and Stretton 1997).

1.2.3.2.2 Other Neuropeptide systems

A host of other peptide neurotransmitters have been detected immunocytochemically in the nematode *A. suum* including pancreatic polypeptide (Brownlee *et al.* 1994), luteinising hormone releasing hormone, small cardioactive peptide B, gastrin, CCK-8a, α-melanocyte stimulating hormone, corticotropin releasing factor, and vasoactive intestinal peptide (Sithigorngul *et al.* 1990, Stretton *et al.* 1991). As yet no data have been published relating to the bioactivity of any of these peptides.

1.3 Nematode neurotransmitter receptors.

The discovery of such a wide range of neuropeptides in the phylum has led to a surge of interest in these systems as potential drug targets, certainly the potency of some of the FaRPs on somatic musculature supports this view, as the established anthelminthics piperazine and levamisole act as paralytics at this site. As no FaRP receptors have yet been cloned from nematodes and none of the existing anthelminthics discovered by direct screening are thought to act through this system, there is no certainty that peptide systems will prove to be valid drug targets. In contrast, $GABA_A$, nAChR and IGluR families of receptors are all validated targets and many subunits of these receptors have been cloned from nematodes.

1.3.1 The "cys-loop" TGIC superfamily.

Vertebrate $GABA_A$, glycine, $5\text{-}HT_3$ and nAChRs, and some of the nematode GABA, glutamate and ACh receptors are all members of the "cys-loop" transmitter gated ion channel (TGIC) superfamily (Barnard 1992 and 1996). The prototype of this class is the nAChR which has been extensively studied in both vertebrates and invertebrates. The receptor from the electric organ of the ray *Torpedo* has been imaged at 9Å resolution and has been demonstated to form a pentameric structure. The five subunits assemble around a central ion channel which is transiently gated by the binding of ACh to the the extracellular region of the receptor (Unwin 1995). A similar pentameric structure has been demonstrated for the $GABA_A$ and $5HT_3$ receptors (Barnard 1996) and implied for the glycine and neuronal nAChRs (*ibid* and Kreienkamp *et al.* 1995). The receptors of this family are predominantly heteromeric (Smith and Olsen 1995, Kreienkamp *et al.* 1995) and it seems likely that this heteropentameric structure is also typical of the nematode members of the class (Cully *et al.* 1994) although this has yet to be confirmed.

The structure of a generic TGIC and a constituent subunit are shown in Figure 6 and Figure 7. The extracellular domain contains residues critical for ligand binding and a ubiquitous cys-loop. The second transmembrane domain (TM2) domain is crucial for ion selectivity (Galzi *et al.* 1992) and in the *Torpedo* nAChR is partially α-helical in nature (Unwin 1995). A similar structure has been postulated for the TM2 of the α_1 subunit of the rat $GABA_A$ receptor (Xu and Akabas 1996). The residues at the mouth of the pore differ markedly between inhibitory Cl^- channels (glycine, $GABA_A$ and glutamate) and excitatory Na^+ channels (ACh and $5\text{-}HT_3$), the former having predominantly positive charged residues and the latter predominantly negative.

The long intracellular loop between the TM3 and TM4 domains is the most variable region of the polypeptide, both in terms of length and amino acid composition. Consensus sequences for phosphorylation by protein kinases are found in this region of many subunits. In vertebrate $GABA_A$ receptors, phosphorylation is an important mechanism for receptor regulation (Swope *et al.* 1992, Gyenes *et al.* 1994, Moss *et al.* 1995). Alternative splicing of some mammalian $GABA_A$ receptor subunit pre-mRNAs yields mature mRNAs encoding subunits which differ only in the intracellular loop region, resulting in differences in protein kinase sites (Harvey *et al.* 1994, Kofuji *et al.* 1991). Alternative splicing of the bovine $GABA_A$ γ_2 subunit pre-mRNA results in two protein products which differ only in the loss of eight amino acids, encoding a protein kinase C (PKC) consensus site, from the shorter of the two subunits (Wafford *et al.* 1991.). The two γ forms were expressed in *Xenopus* oocytes in combination with bovine α_1 and β_1 subunits. Only receptors containing the longer form of the γ_2 subunit exhibited ethanol potentiation of GABA responses, suggesting that phosphorylation of this subunit is necessary for the ethanol potentiation effect (Wafford and Whiting 1992).

1.3.2 The structure of vertebrate inhibitory "cys-loop" TGIC receptors

1.3.2.1 Functional domains of inhibitory "cys-loop" TGIC receptor subunits

Mutagenesis studies on recombinant receptors expressed in *Xenopus* oocytes have highlighted several residues which have a profound effect on GABA affinity. Mutation of a single phenylalanine residue (Phe64) in the putative N-terminal extracellular domain of the rat α_1 subunit drastically reduces GABA affinity for recombinant $\alpha_1\beta_2\gamma_2$ receptors (Sigel *et al.* 1992). An aromatic-glycine-aromatic motif has been shown to be critical for agonist and antagonist binding in both glycine and GABA receptors (Smith and Olsen 1995). Exchange of these two aromatic residues in the α_1 glycine receptor subunit results in a receptor which is gated by glycine, GABA and β-taurine (Schmieden *et al.* 1993).

The characteristic 'cys'-loop was originally considered important for agonist binding (Cockcroft *et al.* 1990), however subsequent mutagenesis studies have shown that it is necessary for efficient subunit coassembly (Amin *et al.* 1994)

Figure 6. Schematic diagram of a generic "cys-loop" TGIC.

Schematic diagram of TGIC in the cell membrane. For clarity, only 3 of the 5 subunits are shown.

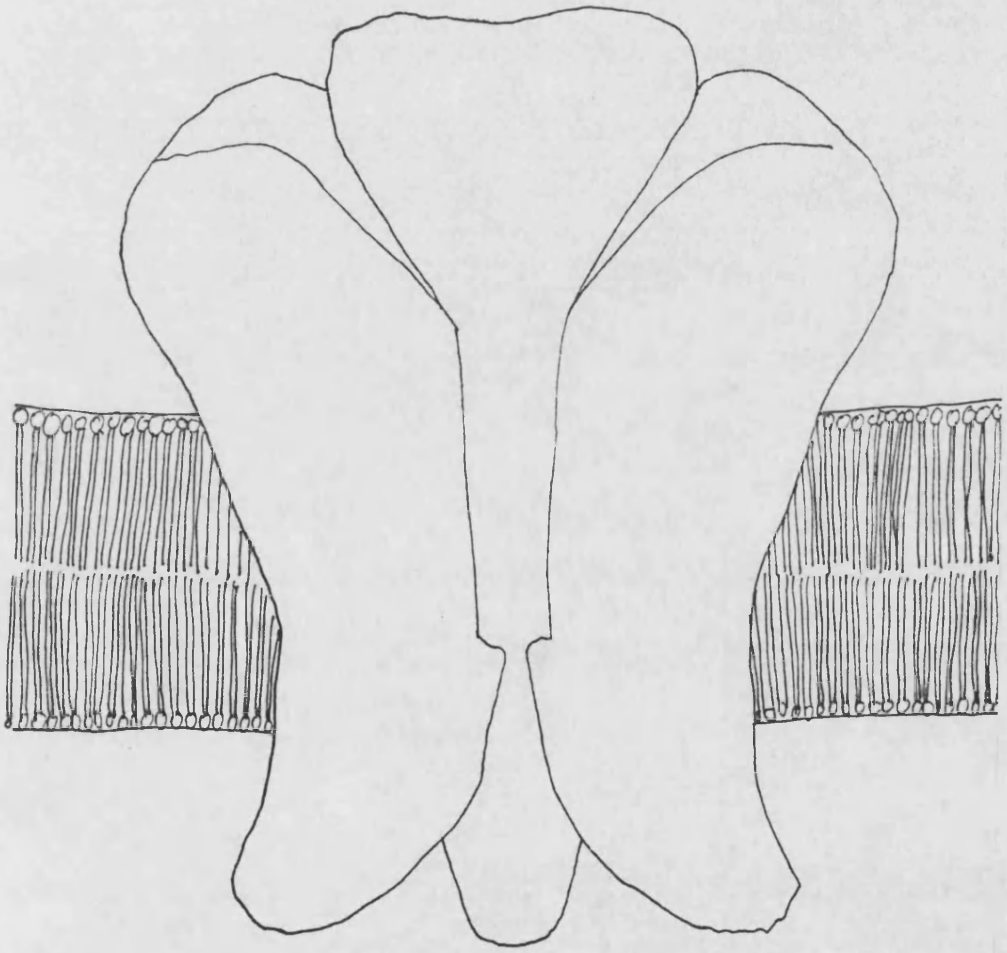
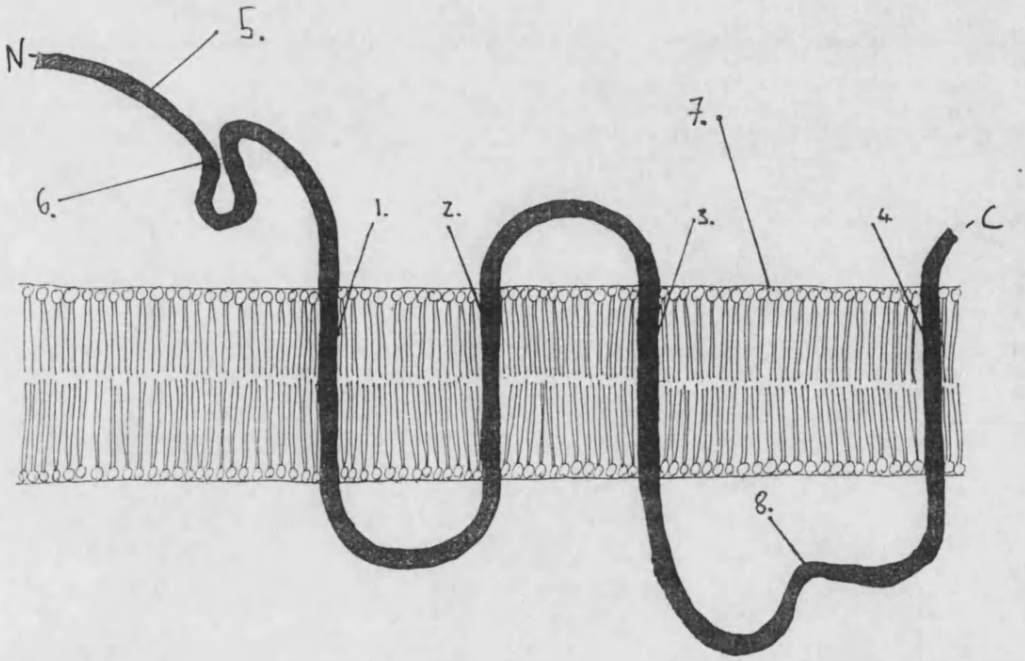


Figure 7 Schematic diagram of a TGIC subunit.

Legend. (*1-4*) transmembrane domains 1-4, (*5*) N-terminal extracellular domain, glycosylation site not shown, (*6*) cysteine loop, (*7*) cell membrane, (*8*) intracellular loop. Not to scale.



The TM2 regions of many vertebrate glycine and $GABA_A$ receptor subunits are highly conserved, having the sequence TTVLTMTT forming the channel lining domain. This region is also implicated in picrotoxin binding (Pribilla *et al.* 1992), a single point mutation of serine to alanine in the TM2 of the *rdl* $GABA_A$ receptor subunit in both the fruit fly *Drosophila melanogaster* and mosquito *Aedes aegyptii* confers resistance to the channel blockers picrotoxin and dieldrin (Ffrench-Constant and Rocheleau 1993, Thompson *et. al* 1993, Chen *et al.* 1994).

1.3.2.1.1 Subunit composition of receptor subtypes.

Molecular cloning of unexpectedly high numbers (over 16 to date) of vertebrate $GABA_A$ receptor subunits has posed some awkward questions concerning which of the multiplicity of possible subunit compositions are found *in vivo*. When possible splice variants are considered, hundreds of thousands of receptor subtypes are possible if the only constraint on receptor formation is a pentameric structure (Barnard 1996).

Published evidence suggests that only a tiny fraction of the possible permutations have a physiological role (MacDonald and Olsen 1994). Sequence comparisons of the cloned receptor subunits reveal subsets of sequences which show a much higher identity with each other than to the remaining subunits. α, β, γ, δ, ε and ρ subunit classes have been defined in this way, the ρ subunits are largely expressed in the retina (Johnston 1994). Naturally occurring isoforms are composed predominantly of 2α, at least one β and at least one γ or δ subunit (Smith and Olsen 1995, Barnard 1996). The role of the recently discovered ε subunit is not known, however it appears to resemble the γ and δ subunits in that it co-assembles with α and β subunits to form functional receptors in heterologous expression systems (Davies *et al.* 1997). mRNAs encoding the α1, β2 and γ2 subunits have been colocalised in parts of the rat brain (Shivers *et al.* 1989). Co-precipitation studies using subunit specific antisera with homogenised brain preparations have supported the αβγ model for the majority of $GABA_A$ receptor subtypes, however their exact stoichemistry is still unknown (McKernan *et al.* 1991, Pollard *et al.* 1991).

1.3.3 "Cys-loop" TGICs in nematodes

1.3.3.1 The cloning of nematode inhibitory TGIC subunits.

Over twenty inhibitory TGIC subunits have been cloned or predicted from genomic sequencing from *C. elegans* and *H. contortus*. Expression cloning of *C. elegans* cDNA yielded two cDNAs encoding subunits of a novel glutamate gated chloride channel (GluClα and GluClβ),

Homomeric expression of the α subunit in *Xenopus* oocytes produced a chloride current of 6μA upon application of 1μM ivermectin 4'-O-phosphate ($IVMPO_4$) with an EC_{50} of 140 ± 15 μM, whereas similar expression of the β subunit gave a 500nA chloride current on application of 1mM glutamate, with an EC50 of 380 ± 20μM. Oocytes expressing both subunits displayed avermectin and glutamate sensitive chloride currents; $IVMPO_4$ concentrations of 1μM or more gated the channels whereas 5nM concentrations were insufficient to cause channel opening but potentiated the response to glutamate. Coexpression produced an upward shift in the EC_{50} for glutamate to 1360 ± 50μM, whereas the EC_{50} for $IVMPO_4$ was little changed. The ranking of potency of avermectin analogues at this recombinant receptor resembles that of their relative potencies as nematocides, suggesting that this is the site of action of this family of drugs. Interestingly, both the EC_{50} and Hill coefficient for $IVMPO_4$ are higher for GluClαβ channels than for those produced by injection of oocytes with *C. elegans* poly $(A)^+$ RNA, suggesting that *in vivo* IGluRs may contain additional subunits (Cully *et al.* 1994).

The GluClβ gene was mapped to chromosome 1 by screening a yeast artificial chromosome (YAC) grid. A probe derived from the 5' end of the GluClβ cDNA was used to identify a 4Kb EcoRI restriction fragment from the cosmid C35E8 which included the upstream genomic sequence. A 2.1Kb PCR product derived from this fragment was sub-cloned into the lacZ reporter fusion construct pPD22.11 and stably transfected into *C. elegans*. The transgenic strains were assayed for β-galactosidase activity, which was observed in the three m4 pharyngeal muscle cells of the metacorpus (Laughton *et al.* 1997). This suggests that GluClβ is a component of a pharyngeal glutamate gated chloride channel and as the avermectins paralyse pharyngeal pumping at therapeutic concentrations (Geary *et al.* 1993), this channel is likely to be the major target for these anthelminthics. The cloning of another GluCl receptor subunit (DrosGluClα) , which shares 48% amino acid identity with *C. elegans* GluClα, has been reported from *D. melanogaster*. Unlike its *C. elegans* orthologue, DrosGluClα produces chloride currents gated by both glutamate and $IVMPO_4$ when expressed homomerically in *Xenopus* oocytes. This subunit, in common with vertebrate glycine receptor subunits and both GluClα and β, has two conserved cysteine pairs in the N-terminal putative extracellular domain (Cully *et al.* 1996). In addition to their anthelminthic activity, the avermectins are potent insecticides. The presence of an avermectin sensitive glutamate gated chloride channel in insects adds further support to the hypothesis that this receptor represents avermectin's major site of action.

Classical genetic studies on uncoordinated (*unc*) mutant *C. elegans* strains and data from the *C. elegans* sequencing project have resulted in the cloning of a $GABA_A$ receptor subunit. *unc*49 mutants show an uncordinated "shrinker" phenotype, characterised by simultaneous contraction of dorsal and ventral muscles. This defect can be produced artificially in N2 individuals by laser ablation of the VD and DD inhibitory motorneurons. The distribution of GABA-immunoreactivity is identical to that seen in wild-type animals, however *unc*49 individuals are resistant to muscimol, a structural analogue of GABA. These experiments show that the *unc*49 gene product is required for postsynaptic GABA function at the neuromuscular junction (McIntire *et al.* 1993a and Reiner and Thomas 1995). The genomic sequence of *unc*49 has been described as part of the *C. elegans* sequencing project and unsurprisingly the predicted gene product shows the characteristic cys-loop motif, agonist binding and transmembrane domains of an inhibitory TGIC subunit (Bamber and Jorgensen 1995).

In this laboratory, a PCR-based approach has been employed to clone inhibitory TGIC subunits from *H. contortus, C. elegans,* and *A. suum*. Degenerate primers were designed to conserved regions in the extracellular and first transmembrane domains of known inhibitory "cys-loop" TGIC receptor subunits and used in the PCR to amplify a heterogenous product of around 0.45kb from *H.contortus* cDNA. Cloning of this product resulted in 5 related inhibitory TGIC subunit partial cDNA clones (Laughton 1993. Laughton *et al.* 1994, 1995). This PCR technique has been applied to the other two species with similar results. Full-length cDNAs for some of these subunits have been cloned using RACE PCR (Random Amplification of cDNA ends - Frohman and Martin 1989, Towner and Gärtner 1992) and inverse PCR (Laughton *et al.* 1994, 1995). The presence of SL1, a common *trans*-spliced leader sequence, at the 5' end of the majority of mRNAs from all three species has facilitated PCR-based cloning methods (Nilsen 1995). The data from sequences cloned using this PCR approach are summarised in Table 1 and Table 2.

Table 1. Putative inhibitory TGIC subunits cloned from C. elegans using PCR-based techniques

Subunit Name	Extent of available information	Probable Native Ligand	References
Ceg1/GluClβ	Full-length cDNA clone (EMBL acc. no. u41525)	Glutamate	Cully *et al.* 1994. Laughton *et al.* 1995
Cegbr2	Full length cDNA clone (EMBL acc. no. U40573)	Glutamate	Laughton *et al.* 1997
Cegbr3	Full length cDNA clone (EMBL acc. no. U41113).	Glutamate	Laughton *et al.* 1997

Table 2. Putative inhibitory TGIC subunits cloned from parasitic species

Species	Subunit Name	Extent of available information	Probable Native Ligand	References
H. contortus	Hg1	Full length cDNA clone (EMBL acc. no.X73584)	?	Laughton *et al.* 1994.
H. contortus	Hg2/3	Partial cDNA sequence lacking only 0.5Kb of 5' terminus	Glutamate	Laughton 1993.
H. contortus	Hg4	Full length cDNA clone (EMBL acc. no.YO9796)	Glutamate	N. Delany, D. Laughton and A. J. Wolstenholme (unpublished results)
H. contortus	Hg5	Partial sequence lacking 3' region.	Glutamate	N. Delany, D. Laughton and A. J. Wolstenholme (unpublished results)
A. suum	Asg2/3	Partial cDNA sequence lacking only 0.2Kb of 5' terminus	Glutamate	S. Jagannathan and A. J. Wolstenholme (unpublished results)

1.3.3.1.1 *Caenorhabditis elegans.*

GluClβ is the only receptor subunit listed in the tables above for which the endogenous ligand is known; in the absence of further expression data the assignment of ligands to the other subunits is speculative and based solely on sequence comparisons.

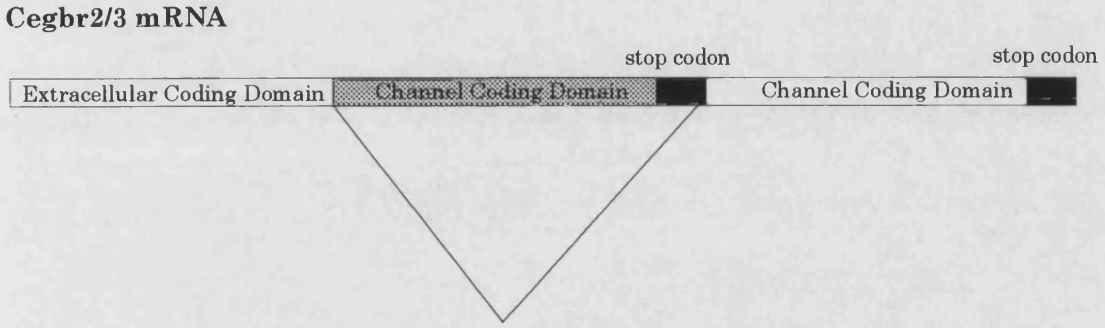
It is likely that Cegbr2 and Cegbr3 are members of the GluCl receptor class as they share 55% and 57% identity respectively with GluClα, moreover the sequence of the predicted protein product contains the extra pair of cysteines in the putative N-terminal extracellular domain, in common with all members of the GluCl class cloned to date (Cully *et al.* 1996). This level of identity is too low for Cegbr2 and Cegbr3 to be α subunits and it is more likely that these are members of a new class of glutamate receptor subunit, GluClγ. The Cegbr2/3 mRNA has an unusual sequence, encoding a complete inhibitory receptor subunit and translational stop codon followed by a sequence which encodes a similar series of 4 transmembrane and 1 intracellular domains. Northern blot analysis of *C. elegans* poly(A)$^+$ RNA with probes derived from the Cegbr2/3 sequence showed that a region of the mRNA encoding the first series of transmembrane and intracellular domains can be spliced out (Figure 8) to yield a mature mRNA which encodes a subunit with the same N-terminal extracellular domain but different channel-forming and intracellular C-terminal regions (Laughton *et al.* 1997). The structure of the Cegbr2/3 cDNA probably arose through a gene duplication event in a common evolutionary ancestor, the possible physiological relevance of this splicing pattern is discussed more fully in 6.3.

Three orthologous cDNAs, or parts thereof, have been cloned in this laboratory from a range of nematode species (*H. contortus* - Hg2/3, *A. suum* - Asg2/3, *C. elegans* - Cegbr2/3). The cDNAs appear to encode subunits sharing high identities (>65%) with Cegbr2/3; the presence *in vivo* of two alternatively spliced transcripts has only been demonstrated in *C. elegans* but it is likely that a similar splicing pattern is found in the other species as the predicted splice sites are well conserved at the nucleotide level. The highly conserved nature of these orthologues suggests that the receptor subunits have important physiological functions and may therefore be potential drug targets.

1.3.3.1.2 *Haemonchus contortus*

The first inhibitory amino acid receptor subunit to be cloned from *H. contortus*, indeed from any parasitic nematode species, was Hg1. When expressed homomerically in the *Xenopus laevis* oocyte expression system, Hg1 formed a glycine gated chloride channel (Laughton *et al.* 1994). The glycine response seen was of low amplitude (10na) and only recorded at relatively high agonist concentrations (>1mM). This was an unexpected result as to date there is no evidence for glycine acting as a neurotransmitter in parasitic or free-living nematodes species.

Figure 8 Alternative splicing pattern of the Cegbr2/3 mRNA.



Homomeric expression of both vertebrate and invertebrate receptor subunits often results in null or nonphysiological responses (Harvey *et al* 1991); a subunit of the *Lymnaea stagnalis* $GABA_A$ receptor exhibited an unusual benzodiazepine response when expressed homomerically in the *Xenopus* system. Diazepam gated the homomeric receptor with a potency over an order of magnitude higher than that of GABA (EC_{50} ~10μM and ~250μM respectively) and the maximal current evoked by diazepam was more than twice that evoked by GABA itself, 35 ± 5 nA and 14 ± 4 nA respectively (Zaman *et al* 1992). Most TGIC isoforms found in vertebrates are heteropentamers and the agonist binding site of most isoforms of both vertebrate acetylcholine and $GABA_A$ receptors is thought to be located at the interface between heterologous subunits (Kreienkamp *et al.* 1995, Smith and Olsen 1995). If a similar model of receptor structure holds for nematodes, homomeric expression of individual subunits may not produce an authentic binding site. Taken together these considerations imply that the expression data for Hg1 may not accurately reflect its native ligand specificity. As the predicted amino acid sequence of Hg1 is most like that of *unc*49, this subunit is also likely to be a component of a $GABA_A$ receptor.

The cDNA encoding Hg2/3, a Cegbr2/3 orthologue, was partially cloned (Laughton 1993) and lacks only 5' terminal sequence. This clone is hereafter called Hg2trun. The predicted amino acid sequences of Hg2 and 3 differ from those of Cegbr2 and 3 in a number of respects, having atypical TM4 regions, as determined by hydropathy analysis and sequence alignments. The Cegbr2/3 cDNA has approximately 100bps of untranslated sequence between the stop codon of Cegbr2 and the splice donor site, this sequence is absent from Hg2trun.

Hg4 shares a high level of identity with GluClβ (80%) and is likely to be the *H. contortus* orthologue of this subunit. Hg5 is a member of the GluClα family of receptor subunits, but is only partially cloned at present, lacking 3' sequence (N. Delany unpublished results).

Data from the C. elegans sequencing project.

The GCG programme FASTA (Devereux *et al.* 1984) was used to search the "Wormpep" and EMBL databases with the predicted amino acid sequences of Cegbr2, Hg1 and GluClα. cDNAs sharing high levels of identity with the query sequence and having the characteristic "cys-loop" motif are presented in both the form of a sequence alignment and tree (Appendix 1 and Figure 9.). The aromatic-glycine-aromatic motif is weakly conserved and preceded by a strongly conserved serine. Given the importance of this motif in defining agonist specificity (Schmieden *et al.* 1993) it is possible that some of these receptor subunits may assemble into receptors gated by unusual

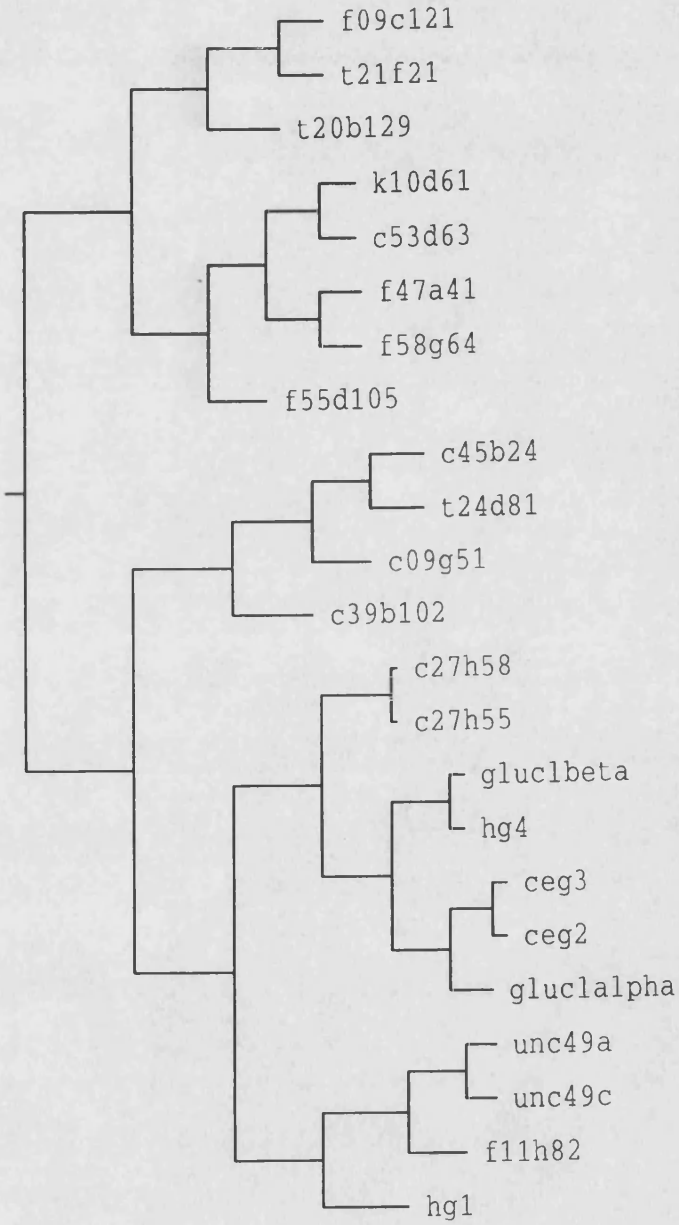
native ligands. The TM2 domains of *unc*49 and F11H8.2 alone have the TTVLTMTT motif which was originally thought to be a defining character of chloride channel subunits (Grenningloh *et al.* 1987). The tree shows that Hg1 and F11H8.2 cluster with the known $GABA_A$ receptor subunit *unc*49. These may all be $GABA_A$ receptor subunits, despite the conflicting expression data for Hg1. Similarly, the cluster containing U14524 and U14525 (GluClα and GluClβ) may all be IGluR subunits. The subunits at the top half of the tree may be components of either of these, or of receptors gated by novel ligands. These inferences are only tentative, as sequence identity alone is not proof of physiological function. It is obvious that the cloning work on these inhibitory TGICs is well progressed, however in contrast to the situation in vertebrates, almost nothing is known about the manner in which these subunits coassemble to form functional receptors *in vivo*. Defining the native isoforms of these subunits and demonstrating their agonist specificities is one of the major tasks in nematode neuroscience. The relative simplicity of the nematode nervous system allows a cell by cell approach to be taken: antisera specific for each subunit can be used in immunocytochemical experiments to define the subset of neurons which express that subunit. Comparison of such distribution patterns will enable the composition of the receptors on each neuron to be described and reconstituted in heterologous expression systems for further study. Comparison of subunit distribution data with the distribution patterns of the classical neurotransmitters will suggest agonist specificities which can be confirmed by these expression studies. Eventual reconstitution of $GABA_A$ and IGluR receptors will give an insight into the structure-function relationships within this class of TGICs and permit the design of mechanism-based high throughput screening programmes for the development of novel anthelminthics.

1.3.3.2 Nicotinic acetylcholine receptors (nAChRs).

The first nematode nAChR subunit cDNAs were cloned using library screening techniques and their functional expression was first reported by Squire *et al.* (1995). A *C. elegans* cDNA library was screened using a probe constructed from a region of the *Drosophila ard* gene, a non-α nAChR subunit. The resulting full-length cDNA clone (acr-2) was co-expressed with the *C. elegans* α subunit *unc*38 in *Xenopus* oocytes. Small (~10na) levamisole-induced sodium currents were observed at levamisole concentrations of ~100μM. Ballivet *et al.* (1996) describe the cloning of two *C. elegans* nAChR subunit cDNAs (Ce21 and Ce13) by screening a library with an avian α_5 subunit cDNA probe. Subsequent expression of Ce21 in *Xenopus* oocytes produced an ACh dependant sodium current (EC_{50} = 55μM) which was antagonised by levamisole (IC_{50} = 36μM). Nicotine acted as an agonist at the homomeric receptor with an EC_{50} of 12.6μM.

Figure 9. Tree showing sequence relationships between putative inhibitory TGICs from C. elegans and H. contortus.

The table of amino identities in appendix 1. is presented as a tree, generated using the GCG program PILEUP (Devereux *et al.* 1984).



The data from the *C. elegans* genome sequencing project are a potent tool for nematode neuroscience. Homology and library searches, combined with genetic analysis of neuronal degradation and levamisole insensitive phenotypes, have indicated that there are at least twenty putative nAChR subunits in *C. elegans* (Mongan *et al.* 1996).

1.4 Aims of this work.

The parasitic worm *H. contortus* was chosen as the main organism of study to maximise the relevance of this research to the design of new anthelminthics. The first aim of this work was to map the expression pattern of Hg1 in adult worms, in order to gain an understanding of its distribution. Localisation studies of other inhibitory receptor subunits are underway in this species (N.Delaney and L. Horoszok, Personal Communication); combining the results of these may allow the design of physiologically relevant co-expression experiments, which circumvent the problems of homomeric expression and give an accurate representation of *in vivo* agonist specificity.

The putative IGluR subunit Hg2/3 has highly conserved orthologues in both *A. suum* and *C. elegans*, suggesting that this subunit may be of significant physiological importance. An Hg2/3 partial cDNA (Hg2trun) has been partially cloned, missing only 5' terminal sequence (Laughton 1993), therefore the other aim of this work was to derive a full sequence for the Hg2/3 cDNA.

2. Materials

2.1 *Escherichia coli* strains & plasmids

STRAIN	GENOTYPE (see Appendix 2.)	SUPPLIER
XL1-Blue	SupE44, hsdR17, recA1, endA1, gyrA46, thi, relA1 lac-, F [ProAB+, LacIq, LacZ, delM15, Tn10(tetR)]	Invitrogen, Abingdon
XL2	SupE44, hsdR17, recA1, endA1, gyrA46, thi, relA1 lac-, F [ProAB+, LacIq, LacZ, delM15, Tn10(tetR) Amy Cam^r]	Invitrogen
TB1	F- ara- Ð(lac-proAB) rpsL (Str^r)[Ø80dlacÐ(lacZ)M15]thi hsdR($r_k^- m_k^+$)	New England Biolabs, Hitchin

PLASMID	GENOTYPE AND SIZE	SUPPLIER
pBluescript™	Amp^r, 2.96kb	Stratagene, Cambridge
pMAL-C2	Amp^r, 6.7kb	New England Biolabs

2.2 Enzyme Buffers

ENZYME	COMPOSITION (AT 1X)
Polynucleotide kinase (PNK)	70mM tris-HCl (pH7.6),10mM $MgCl_2$,5mM DTT
Taq DNA polymerase	50mM KCl,10mM tris-HCl (pH9.0),0.1% Triton X-100
Xpand enzyme mix	50 mM tris-HCl, pH 9.2, 16mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$
Restriction Enzymes	10mM tris-acetate (pH 7.5), 10mM MgOAc, 50mM KOAc
Alkaline Phosphatase	50mM Na Cl,10mM tris-HCl (pH7.9),10mM $MgCl_2$,1mM DTT
T4 DNA Ligase	50mM Tris-HCl (pH 7.2), 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 50μg/ml BSA - pH 7.8

2.3 Nematode Tissue

H. contortus eggs were supplied by Pfizer laboratories in Sandwich, Kent and Dr. Gerald Coles, Bristol University. Adult *H. contortus* were supplied by Dr. Coles. Adult *A. suum* were supplied by Dr. R. Martin, Edinburgh and Dr. L. Holden-Dye, Southampton.

2.4 Enzymes

Restriction enzymes, T4 DNA ligase, polynucleotide kinase and associated buffers were supplied by New England Biolabs. T7 DNA polymerase, "Sequenase™" 2.0 DNA sequencing kit, "Sequenase™" PCR product sequencing kit and "Sequenase™" quick denature plasmid sequencing kit were all purchased from Amersham International, Little Chalfont. "Sequagel™" sequencing gel reagents were supplied by Flowgen, Lichfield and all radiochemicals were provided by Amersham International.

2.5 General Laboratory Reagents

Other laboratory reagents were bought from Sigma, Poole and BDH, Lutterworth.

2.6 Media

MEDIUM	COMPONENTS g L^{-1}
LB	10g bacto-tryptone, 10g NaCl, 5g yeast extract
LB agar	As above with the addition of 15g agar
DY	16g bacto-tryptone, 5g NaCl, 10g yeast extract
Rich Broth	20g bacto-tryptone, 1g NaCl, 5g yeast extract, 2g $MgCl_2$, 3g glucose ($MgCl_2$ and glucose added post-autoclaving as 2M filter-sterilised stock solutions).

2.7 Standard Reagents

REAGENT	COMPONENTS
10xTBE	0.89M tris-HCl, 0.89MH_3BO_3, 20mM EDTA, pH8.0
Phenol/chloroform	50% Phenol equilibriated with 10mM tris-HCl, pH7.6. 49 % chloroform, 1% iso-amyl alchohol.
PBS	140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4 pH7.4
TE	10mM tris-HCl, 1mM EDTA, pH 7.6

2.8 Antiserum production and characteristation

Peroxidase conjugated goat anti-rabbit serum, thyroglobulin, tetramethylbenzidine, dimethylsulphoxide, dithiothreitol and *m*-maleimidobenzoic acid *N*-hydoxysuccinamide ester (MBS) were purchased from Sigma. Biogel P-2 was supplied by Biorad Ltd, Hemel Hempstead. Cyanogen Bromide activated Sepharose™ was from Pharmacia, St. Albans.

2.8.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

REAGENT	COMPOSITION
SDS-PAGE sample buffer	50mM tris HCl (pH6.8), 100mM dithiothreitol (DTT), 2%SDS, 0.1% bromophenol blue, 10% glycerol
Tank Buffer	25mM tris H-Cl, 250mM glycine, 0.1%SDS pH8.3
Coomassie Stain	2.5mgml^{-1} Coomassie brilliant blue in destain
Destain	45%CH_3OH, 45% water, 10% acetic acid

2.8.2 Western blotting and immunodetection

BUFFER	COMPOSITION
Transfer Buffer	39mM glycine, 48mM tris (pH 8.3), 0.037% SDS, 20% CH_3OH
Blocking buffer	5% Marvel™ non-fat milk powder in PBS, 1mgml^{-1} NaN_3, 0.5% Tween
Washing buffer	PBS, 1mgml^{-1} NaN_3, 0.5% Tween

2.8.3 ELISA (Enzyme-Linked Immunosorbent Assay) Reagents

REAGENT	COMPOSITION
Coating buffer	15mM Na_2CO_3, 35mM $NaHCO_3$, 0.01% NaN_3 (pH 9.6)
Washing buffer	PBS, 0.1% Tween™
Stock acetate/citrate buffer	1M sodium acetate (NaOAC), 30mM trisodium citrate ($Na_3C_6H_5O_7$) pH6.0
Stock TMB solution	10mgml^{-1} TMB in DMSO
Substrate solution	5% stock acetate,citrate buffer, 1% stock TMB solution, 0.006% hydrogen peroxide
Stop solution	1.84M H_2SO_4 (1:10 dilution of 98% stock)

2.9 Immunocytochemistry

TRITC- and FITC- conjugated goat anti rabbit IgG, collagenase type IA and n-propylgallate were supplied by Sigma.

2.9.1 Immunocytochemistry reagents

REAGENT	COMPOSITION
Artificial Perienteric Fluid	67mM NaCl, 67mM $NaCH_3COO$, 3mM CaCl2, 16mM $MgCl_2$, 3mM KCl, 5mM tris-acetate (pH 7.6)
PFA/Glutaraldehyde composite fixative	3.7% Paraformaldehyde,1% glutaraldehyde
Antibody dilution solution	0.1%BSA,0.5%Triton X-100,0.05%NaN_3 in PBS
PBS/Triton	PBS,0.1% Triton X-100
Mounting medium	0.1M n-propylgallate,80% glycerol
Storage buffer	0.1% PFA in PBS
BME Solution	5% β-mercaptoethanol,1% Triton X-100,125mM tris pH6.9
Collagenase buffer	1mM CaCl,100mM tris pH7.5
Cryoprotection Solution	PBS,30% sucrose

2.10 Cloning of the 5' end of the Hg2/3 cDNA

Dynabeads™ were from Dynal Ltd, Norway. Superscript™ reverse transcriptase was supplied by Gibco, Paisley. *Taq* DNA polymerase and associated reaction buffers were purchased from Promega, Southampton.

2.10.1 Southern blotting reagents

REAGENT	COMPOSITION
Denaturation Solution	1.5M NaCl, 0.5M NaOH
Neutralisation Solution	1.5M NaCl, 0.5M tris-HCl pH 7.2, 1mM EDTA
20xSSC	3M NaCl, 0.3M $NaCH_3COO$, pH7.0
100xDenhardt's	2% w/v ficoll, 2% w/v polyvinylpyrrolidone, 2% w/v bovine serum albumin.
Prehybridisation solution	6xSSC, 5xDenhardt's solution, 0.5% SDS, $20\mu gml^{-1}$ heat denatured salmon sperm DNA
Wash solution	2xSSC, 0.1% SDS

2.10.2 Reagents for mRNA purification and cDNA synthesis

REAGENT	COMPOSITION
RNA Extraction	
Denaturation Solution	4M guanidinium thiocyanate, 25mM $Na_3C_6H_5O_7.2H_2O$ pH 7.0, 0.5% sarcosyl, 0.1M β-mercaptoethanol
mRNA Purification and cDNA Synthesis	
2x binding buffer	20mM tris-Cl pH7.5, 1M LiCl, 2mM EDTA
Washing buffer	10mM tris-Cl pH 7.5, 0.15M LiCl, 1mM EDTA
10x first strand synthesis buffer	250mM tris-HCl (pH 8.3), 375mM KCl, 15mM $MgCl_2$
STE	10mM tris-HCl (pH8.0), 1mM EDTA, 100mM NaCl
Alkaline electrophoresis buffer	300mM NaOH, 20mM EDTA
Alkaline electrophoresis loading dye	20%glycerol, 25mM NaOH, 0.02% bromophenol blue

3. Methods

3.1 General Methods

3.1.1 Purification of *H. contortus* eggs from sheep faeces.

Lambs which were free from helminth infection were experimentally infected with L3 *H. contortus* isolates from a wild population susceptible to all known anthelminthics and maintained in a helminth-free indoor environment. Approximately 3 weeks post-infection, faeces from infected sheep were collected in polythene nappies. This work was done at the Department of Veterinary Medicine, University of Bristol by G. Coles. Stools were homogenised in a blender with enough water to form a thin paste which was then washed through a 150μm sieve and the filtrate retained. The liquid was centrifuged for 2 minutes at 1500rpm and the pellet resuspended in saturated sodium chloride solution. Falcon™ tubes (50ml) were filled with this suspension until a meniscus formed over the top of the tube. A large glass coverslip was placed over the top to give a tight seal. The tubes were then centrifuged again at 1000rpm for 2 minutes after which the coverslips were removed quickly. The film of eggs remaining on the liquid covering the coverslip was washed off into a fresh Falcon tube. The eggs were used immediately or weighed into cryotubes and frozen in liquid nitrogen for long-term storage.

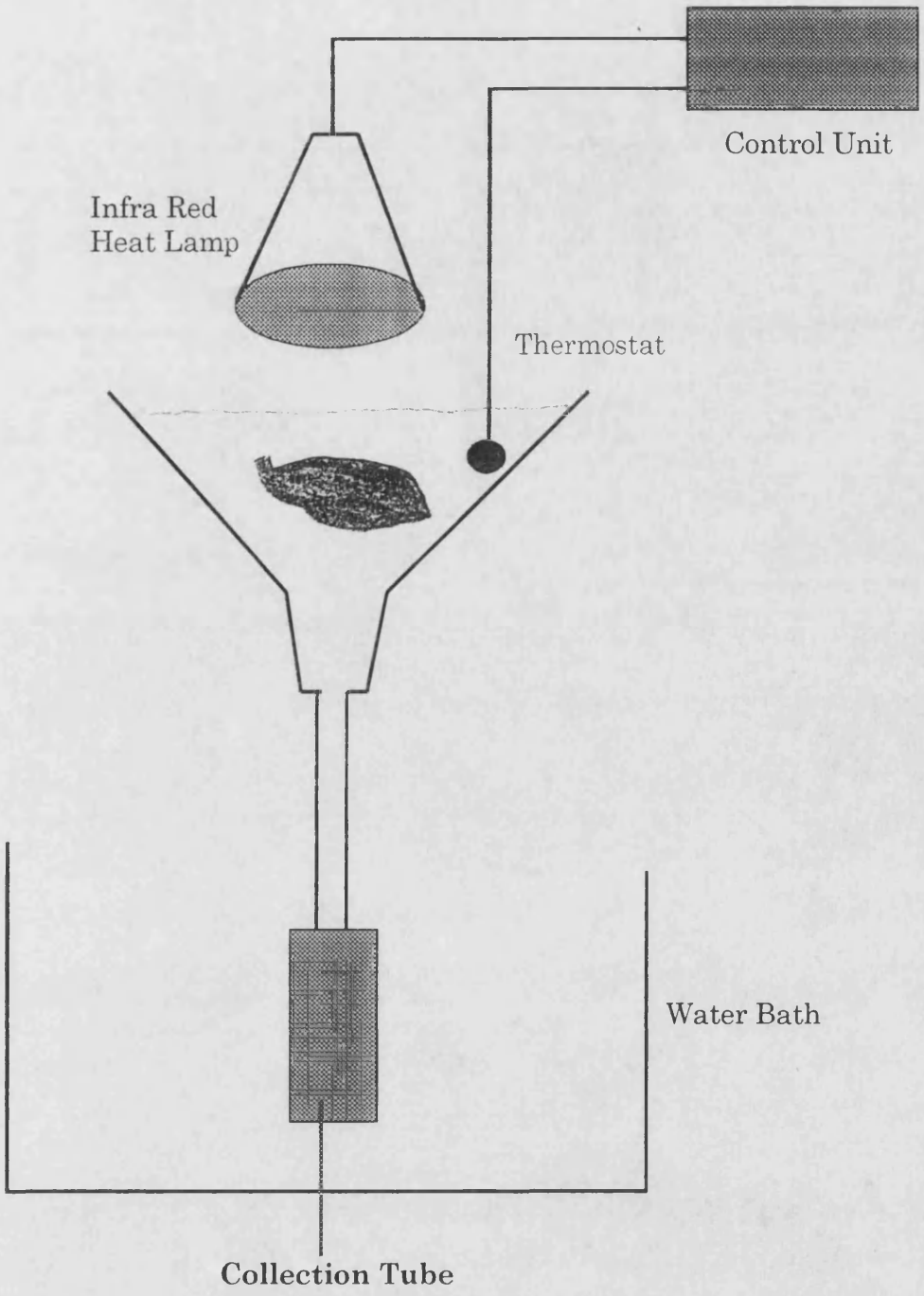
3.1.2 Isolation of adult *H. contortus*

Infected sheep were slaughtered and the intestines removed during butchering by abbatoir staff. The abomasum was cut open and semidigested contents removed by gentle rinsing in water. Adult *H. contortus* could be seen attached to the wall of the abomasum (Figure 10). The cleaned organ was placed in the isolation apparatus (Figure 11). The water bath and thermostat were set at 37°C and the funnel and collection tube filled with pre-warmed PBS. The live adults collected were placed immediately into fix or liquid nitrogen depending on whether they were to be used for immunocytochemistry or RNA extraction.

Figure 10. Post-mortem abomasum from infected sheep showing H. contortus. adults attached to stomach wall.

Scale: approximately life size

Figure 11. Apparatus used for collecting live worms from abomasi.



3.1.3 Ethanol precipitation of DNA.

DNA was precipated out of aqueous solution by adding 0.1 volumes of 3M NaOAc (pH 5.6) and 3 volumes of absolute ethanol. The tube was chilled at -20^{o}C for at least 1 hour, then centrifuged at full speed (14,000rpm) for 30 minutes in a chilled benchtop microcentrifuge. Salt was removed by washing the pellet in 500µl of 70% ethanol and the pellet dried under vacuum to remove all traces of ethanol. The pellet was then resuspended in an appropriate amount of water or TE.

3.1.4 Phenol extraction

An equal volume of phenol/chloroform/iso-amyl achohol (50:49:1) was added to the sample and mixed by vortexing. The tube was centrifuged for 2 minutes at 5,000g and the upper aqueous phase retained.

3.1.5 Purification of DNA using the "Qiafilter™" system

The Qiagen "QIAquick"™ method was used to purify DNA fragments of 100bps or larger from solutions containing contaminating DNA fragments, glycerol, salts or enzymes. PB buffer (5 volumes) was added to the DNA solution and the mixture loaded onto a QIAquick column. The column was centrifuged for 2 minutes at 10,000g and the eluate discarded. Buffer PE (750µl) was loaded onto the column which was centrifuged as before and the eluate again discarded. The last traces of wash buffer were removed by a final spin for 1 minute at 10,000g. The purified DNA was eluted from the column by the addition of 30µl of water and incubation for 2 minutes prior to a final centrifugation at 10,000g for 2 minutes.

3.1.6 Agarose gel electrophoresis of DNA

Agarose gels were made up by adding 1-4% agarose to TBE buffer. The agarose was dissolved by bringing the mixture slowly to the boil in a microwave oven. Ethidium bromide was added to the solution to a final concentration of 50ngml^{-1} as a fluorescent stain.

Gels were poured in perspex gel rigs and allowed to set at 4°C for one hour. The comb was then removed and the gel placed in a tank of TBE buffer. Loading dye (1/5th of a volume) was added to each of the samples which were then loaded into the gel. Gels were typically run at 6Vcm-1

for an hour prior to visualisation under UV illumination. Phageλ DNA cut with the restriction enzyme PstI was used as a size marker.

3.1.7 Purification of DNA from agarose gels

The band of interest was cut from the gel with a clean razor blade and any excess agarose trimmed away. The gel slice was weighed and 250µl (or 1µl for each mg of agarose, whichever was the greater) of gel solubiliser (buffered NaI) added. The agarose was dissolved by incubating the tube at 60 ^{o}C for 10 minutes. Sephaglas BP (5µl of 20% Sephaglas in aqueous solution or 5µl per estimated µg of DNA present) was added and the tube mixed by vortexing. The sample was incubated on the bench for a further 5 minutes with additional periodic vortexing. The Sephaglas was centrifuged at top speed in a microcentrifuge for 1 minute and the supernatant aspirated. Wash buffer (40 µl or 8x the volume of Sephaglas used, of 60% ethanol 20mM Tris-HCl pH 8.0, 1mM EDTA, 100µM NaCl) was added and the pellet resuspended by vortexing. The Sephaglas was centrifuged again as before and the supernatant aspirated. This step was repeated twice. The pellet was air dried for 10 minutes before eluting the DNA by adding a suitable volume of elution buffer(10mM Tris-HCl, 1mM EDTA), not less than half the volume of Sephaglas used. The sample was vortexed briefly to resuspend the pellet and incubated at room temperature for 5 minutes, then centrifuged again for 1 minute at top speed before the supernatant was carefully removed and retained.

3.1.8 Restriction digestion of DNA

DNA samples of 1-5 µg were digested with one or more chosen restriction enzymes as follows

2 µl	10X restriction enzyme buffer
1µl	Enzyme (typically 10U/µl)

Plasmid DNA and H_2O to 20 µl

The enzyme buffer used depended on the enzyme in use. Double digests were performed in "1PhorAll"™ buffer. Single digests were carried out in the manufacturer's recommended buffer; digests were incubated at 37°C for 1-12 hours. If further enzymatic treatment of the sample was required and the restriction enzyme used was susceptible to heat denaturation, the sample was incubated at 65°C for 10 minutes. When using thermostable enzymes an additional "Qiaquick" purification step was used instead.

3.1.9 DNA Ligation

Approximately 200ng of vector was used in each 20µl ligation reaction. A series of reactions was set up with molar insert:vector ratios of from 1:1 to 5:1. Two vector only controls were included, one without ligase. Polyethylene gycol was added when performing blunt-ended ligations to a final concentration of 5% (w/v). The reactions were incubated overnight at 16°C. A typical reaction mixture was as follows;

For a 3:1 insert :vector ratio -	
Vector (pBluescript - 2.96kb)	200ng
Insert (600bp)	120ng
25% polyethylene glycol 8000	4µl
10Xligase buffer (NEB)	2µl
T4 DNA ligase (1unit ml^{-1})	1µl
H_2O	to 20µl

3.1.10 Production of competent cells

Competent cells were made using the calcium chloride method (adapted from Mandel and Higa 1970). An Erlenmeyer flask containing 50 mls of sterile DYT was seeded with 0.7 mls of a fresh universal culture of XL1s or TB1s in a 250ml. The culture was incubated at 37°C with shaking until the OD_{330} was around 0.3. The culture was then centrifuged at 4°C and 3000rpm for 10 minutes and resuspended in 20ml of ice-cold 100mM calcium chloride. The suspension was left on ice for 20 minutes before another centrifugation at 3000rpm for 5 minutes. On completion of the centrifuge run, the cells were immediately resuspended in 4ml of the above calcium chloride solution. Competent XL1s were used immediately whereas TB1s were used within 24hrs.

3.1.11 Transformation and culture of clones

Samples (300µl) of the cell suspension were aliquoted into 1.5ml microfuge tubes which had previously been chilled on ice. A fraction (5µl) of the ligation mixture above (diluted into 50µl if PEG was used in the ligation reaction) was added to the cells and the tubes mixed and left on ice for 40 minutes. After a 1.5 minute heat shock at 42°C, 0.7mls of DYT was added to each tube. The cells were allowed to recover for 1 hour at 37°C, before being plated out onto LB agar containing 50µgml^{-1} ampicillin. After 14-16 hours incubation at 37°C, the plates were

examined, and individual colonies lifted and transferred aseptically to 10mls of LB containing 50 μgml^{-1} ug ampicillin. The cultures were incubated overnight at 37°C with shaking.

3.1.12 Mini-preparations of plasmid DNA using the alkaline lysis method

Small amounts (~10μg) of plasmid DNA were isolated from cultures using a modified alkaline lysis method (Birnboim and Doly 1979). Samples (1.5mls) of an overnight culture were centrifuged for one minute, and the supernatants removed. The pellets were resuspended, with mixing, in 100μl of TEG (50mM glucose, 10mM EDTA, 25mM Tris Cl pH 8.0) and 200μl of fresh 0.2M NaOH 1%SDS. The tubes was incubated on ice for 5 minutes, before 150μl of chilled potassium acetate (3M K^+ 5M OAc^- was added) and the tube vortexed. After a further 5 minutes on ice the tubes were centrifuged at 15,000 rpm for 5 minutes and the supernatants preserved. DNA was then extracted once with phenol:chloroform and precipitated for 15 minutes at -20°C with 2 volumes of ethanol. After a 10 minute centrifugation the pellets were washed with 70% ethanol and dried under vacuum before resuspension in TE containing 10μg ml^{-1} RNAseA.

3.1.13 Mini-preparations of plasmid DNA using the "Wizard™" Miniprep kit

Samples (3ml) of an overnight culture were centrifuged in a benchtop microcentrifuge in two microfuge tubes at 10,000g for 90 seconds. Each of the pellets was resuspended by vortexing in 100 μl of resuspension solution (50mM tris-HCl pH7.5, 10mM EDTA, 100μg/ml RNase A) and the two aliquots combined. Cell lysis solution (200μl of 0.2M NaOH, 1% SDS) was added and the tube's contents mixed by inverting several times. After the addition of 200μl of neutralisation solution the tube was again inverted several times and centrifuged at top speed (14,000rpm) in a microcentrifuge for 5 minutes. The clear supernatant was retained and the pellet of bacterial debris discarded.

Wizard™ miniprep DNA purification resin (1ml) was added to the supernatant with gentle mixing and a Wizard™ minicolumn was prepared by attaching the barrel of a 3ml syringe to the minicolumn. The resin/supernatant mixture was pipetted into the column and the plunger pushed home gently, after which the syringe was removed from the column and the plunger withdrawn. The syringe barrel was then reattached and 2ml of column wash solution was passed through the minicolumn. The syringe was again removed and the minicolumn transferred to a clean microfuge tube. The minicolumn was centrifuged at 10,000g in a microcentrifuge for 2 minutes

to dry the resin. DNA was eluted by adding 50 µl of water and centrifuging at 10,000g for 30 seconds.

3.1.14 Synthesis and deprotection of Oligonucleotides

Oligonucleotides were synthesised in house using the phosphoramidite method and deprotected by emptying the cartridge into a screw top tube containing 1ml of fresh concentrated ammonia solution. The tube was incubated at 55°C overnight, cooled, opened carefully to avoid spillage and transferred to a 15ml Corex tube. The solution was neutralised gradually with 1ml acetic acid and precipitated by adding 6mls of absolute ethanol. The pellet was resuspended in 1ml of water and removed from the phosphoramadite precipitate. The yield was calculated by adding 5µl of purified oligonucleotide solution to 995µl of water in a quartz spectrophotometer cuvette. The OD_{260} was measured against a water blank using a spectrophotometer and the amount of DNA present estimated according to the following equation :-

DNA present (in µgs) = 33 x OD_{260}

3.1.15 Sequencing using the "Sequenase™" quick denature plasmid sequencing kit.

Plasmids were sequenced using the chain termination method (Sanger *et al.* 1977) according to the instructions supplied by the manufacturers with the kit.

3.1.16 Preparation of polyacrylamide gels for sequencing

The gels were cast in rigs supplied by Flowgen. Both glass plates were washed in water and polished with 95% ethanol. The back plate was laid flat and coated in a mixture of 200µl silane, 25ml absolute ethanol and 1.2mls 10 % acetic acid. After 5 minutes the plate was rinsed first with water then with ethanol and polished. The front plate was similarly coated in 1% dichlorodimethylsilane in 1,1,1, trichloroethane, left for five minutes, washed with water and polished with 95% ethanol. The gel rig was assembled with 0.2mm spacers and a gel mixture of the required acrylamide concentration was made up, adding 300µl of fresh 16% ammonium persulphate and 30 µl of temed just before pouring. For direct sequencing of PCR products a glycerol tolerant buffer was used to improve band definition The gel was poured in a horizontal position and the reverse (flat) face of a 0.2mm thick shark's tooth sequencing comb inserted into the upper edge of the gel. The acrylamide was allowed to set for an hour before the comb was removed and reversed so that the teeth impinged about 1.5mm into the surface of the gel.

The gel rig was mounted vertically in the equipment supplied and the reservoirs filled with 1XTBE. The wells were washed clear of urea and any loose polyacrylamide fragments with a pasteur pipette to prevent interference with the DNA samples. A fraction (3µl) of each sample was loaded onto the gel which was run at a limiting 45W. The run was terminated after approximately 45 minutes when the first blue dye had reached the bottom of the gel. After the run, the two plates were separated and the back plate with attached gel was fixed with 10% methanol, 10% acetic acid for 10 minutes then rinsed under a gentle flow of water for a further 10 minutes. After drying in an oven at 90°C the gel was autoradiographed.

3.2 Preparation and characterisation of the polyclonal antiserum anti-HGP1

3.2.1 Preparation of the synthetic antigen HGP1-thyroglobulin

3.2.1.1 Peptide Synthesis

The peptide was synthesised automatically using fmoc synthesis on a Milligen 9050 Pepsynthesizer. This work performed by Dr. Kinsman and Peter Jenkins, University of Bath.

3.2.1.2 Congugation of HGP1 to a carrier protein

A 20mgml^{-1} solution of thyroglobulin in 10mM potassium phosphate pH 7.2 was dialysed overnight against 10mM potassium phosphate pH 7.2 and the final solution adjusted to a concentration of 16mgml^{-1}. A 10mgml^{-1} solution of the peptide in 10mM potassium phosphate pH 7.2 was reduced for one hour at room temperature with a final concentration of 200mM DTT. The peptide was then desalted on a Bio-Gel P2 column (Pharmacia). Thyroglobulin solution (250µl) was mixed end over with 85µl of MBS (3mgml^{-1} in dimethylformamide) for 30 minutes at room temperature.

The thyroglobulin was desalted on a Pharmacia P30 column equilibriated with 50mM potassium phosphate pH7.2, and collected as three 1ml fractions. The three fractions were combined and 1ml of the peptide solution added (adjusted to 5mgml^{-1}) and conjugation was allowed to proceed for three hours at room temperature, with constant mixing.

3.2.2 Production and adsorbtion of antiserum

3.2.2.1 Innoculation and Bleeding

NaCl (0.9%) was added to the conjugated mixture to adjust the osmotic potential of the inoculation mixture to a physiological level. This mixture (250µl) was combined with the same volume of Hunter's Titermax adjuvant and the mixture was passed repeatedly between two connected 2ml syringes to ensure adequate emulsification. New Zealand white rabbits were innoculated subcutaneously in the hind leg. An ear bleed was performed after 9 days. All animal handling was done by licensed animal house staff. The titres of each serum against both HGP1 and thyroglobulin were assayed using the ELISA. Exsanguination was performed by animal house staff using cardiac puncture of anaesthetised rabbits and yielded approximately 40mls of serum.

3.2.2.2 Harvesting of serum

Collected blood was transferred to a 50ml Falcon™ tube and allowed to clot for one hour at 37°C then incubated at 4°C overnight. The clot was freed from the sides of the tube with a sterile pippette tip and the sample centrifuged at 1500 rpm for 20 minutes. The supernatatant was removed and 0.1% sodium azide added as a preservative. Aliquots for long term storage were stored at -70°C, short-term storage was at 4°C.

3.2.2.3 Adsorbtion of antibodies against thyroglobulin

3.2.2.3.1 Coupling of thyroglobulin to CNBr Activated Sepharose.

Thyroglobulin (50mg) was dissolved in 8mls of coupling buffer (0.5M NaCl 0.1M $NaHCO_3$ pH 8.3). CNBr activated Sepharose (1.5g) was washed for 15 minutes with 300 mls of 1mM HCl on a sintered funnel and then added to the thyroglobulin solution. The mixture was stirred end over end for one hour at room temperature, then washed with 30 mls of coupling buffer.

The gel was soaked for two hours in 0.1M Tris-HCl pH 8.0, before three cycles of washes in alternating pH. Each cycle consisted of a wash in 30 mls of 0.1M acetate pH 4.0/0.5M NaCl followed by 0.1M Tris-HCl buffer pH 8.0/0.5M NaCl.

3.2.2.3.2 Adsorbtion of antiserum on a thyroglobulin column

The gel slurry was loaded into a 5ml syringe blocked with silanised glass wool. The serum was filtered through a 0.22µM filter to remove particulates before application to the column, the

filtered serum was cycled through the column for two hours at room temperature. The column was washed with 50 mls of 50mM glycine/HCl pH 2.3 before undergoing three cycles of alternating pH washes as above. The regenerated column was then re-used. Aiquots (2ml) of sera were treated with sodium azide to a final concentration of $1mgml^{-1}$ and stored at -20°C until required.

3.2.3 Confirmation of antiserum affinity for recombinant Hg1(ex)

3.2.3.1 Production of Hg1(ex) fusion protein construct.

Approximately 100pg of an Hg1 cDNA clone in pBluescript sk/ks was used as template in the PCR with the primers MalS and MalA. These primers are complementary to target sequences flanking the cDNA sequence which encodes the extracellular region of Hg1 (Hg1[ex]). Both primers incorporated unique restriction sites which allowed ligation of the resulting PCR product into the multiple cloning site of the expression vector pMAL-C2 in the correct reading frame.

OLIGONUCLEOTIDE	SEQUENCE - restriction site in bold, gene specific sequence underlined.
MalS- sense primer (BamHI)	5'GACAGC**GGATCC**ATGAACCTTTTCTTCGT 3'
MalA- antisense primer (XbaI)	5'AATGCG**TCTAGA**ATTAACCCTGTTGGCGTTGCAGCT 3'

PCR amplification was performed using the "Xpand™" high-fidelity PCR system to minimise the probability of PCR induced mutation. The reactions were set up as shown below.

5µl	10XReaction Buffer
5µl	4X dNTPs (4mM)
3µl	MalA Primer (6.5µM)
3µl	MalS Primer (6.5µM)
1µl	template (Hg1 in pBluescript $10pg\mu l^{-1}$)

Water to 50µl.

The reaction mix was overlayed with sterile mineral oil in a 0.5ml microfuge tube and heated to $93^{o}C$ for 2 minutes to destroy any secondary structure in the cDNA.

DNA polymerase mix (4 units) was added and the reaction mixture was thermally cycled through 35 rounds of:-

Denature	30sec	93°C
Annealling	1min	53°C
Extension	1min30sec	72°C

Final extension was at 72°C for 7minutes. The PCR products were electrophoresed through a 1.2% agarose gel and the band of interest cut out and Sephaglas purified. The purified product was digested for 3 hours at 37°C with 20 units each of XbaI and BamHI in "One-Phor-All" buffer. 5 μgs of pMAL-C2 vector was similarly restricted in a parallel reaction. Both the restricted products were purified on "Qiaquick" columns and ligated together as described above. Ligation products were initially transformed into XL2s, wizard minipreps of transformants were used to transform competent TB1 cells, made using the calcium chloride method. TB1 transformants were used for expression.

3.2.3.2 Expression using pMAL vectors

Samples (0.8ml) of an overnight culture of TB1s transformed with Hg1(ex) in pMAL were innoculated into a 100ml culture of Rich Broth containing 100μgml-1 ampicillin. Each culture was incubated at 37°C with shaking to an absorbance at 600nm of 0.5 (approximately 3 hours). 1ml of culture was taken and centrifuged at maximum speed in a microcentrifuge tube for 2 minutes. The supernatant was discarded and the pellet resuspended in 100μl of SDS-PAGE sample buffer prior to storage at -20°C.

IPTG was added to a final concentration of 300μM and the culture incubated for a further two hours. A 0.5ml sample of induced culture was taken and processed as above.

3.2.3.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

3.2.3.3.1 Sample preparation

Samples were sonicated on ice for 30 seconds to rupture the plasma membrane and release protein. These samples were then denatured by incubation in a boiling water bath for 5 minutes, immediately prior to loading onto the gel.

3.2.3.3.2 Preparation and running of 12% polyacrylamide gels

Gels were prepared by combining 2.5mls of 1.5M Tris pH8.8, 4mls of "Protogel™", 100µl 10% SDS , 40µl of 25% ammonium persulphate and 4µl of TEMED in a total volume of 10mls, this mixture was then poured into an "Atto™" minigel rig. Gels were overlaid with water-saturated butan-1-ol and allowed to polymerise for 20 minutes. The butan-1-ol layer was discarded and the comb inserted between the gel plates. 5% stacking gels were made by combining in a total volume of 8mls; 1.0ml of 1M Tris pH6.8, 1.3ml protogel, 100µl 10% SDS, 40µl of 25% ammonium persulphate and 10µl of TEMED. This was poured onto the resolving gel and allowed to set for a further 40 minutes. 10µl of each sample was loaded into each well and gels were run in tank buffer at 175 volts for 90 minutes.

3.2.3.4 Western transfer to nitrocellulose membranes.

Three gel sized pieces of Whatman 3MM filter paper were soaked in transfer buffer and aligned on the graphite transfer plate. Gels were removed from the electrophoresis rig, rinsed briefly in water, and rolled out over the filter paper surface, eliminating any air bubbles. A piece of nitrocellulose membrane, previously cut to size , was soaked in transfer buffer and placed on top of each gel. Three more pieces of buffer-soaked filter paper were put on top of the membrane and the upper graphite plate lowered on top of the stack. The transfer rig was connected with the cathode at the base and a current of 20mA passed through the blot for 2 hours. Gels were visualised by incubating in Coomassie stain at room temperature overnight followed by 8 hours in destain solution.

3.2.3.5 Immunodetection of bound protein

Detection was performed using the horseradish peroxidase chemiluminescent detection method which exploits indirect immunodetection with a peroxidase conjugated secondary serum. Detection relies on the oxidative action of peroxide on luminol, a cyclic diacylhydrazide. The activated oxidised form of luminol decays back to its ground state with the concommitant emission of light. The ECL kit was used which also contains enhancers such as phenols, in the presence of these agents both the intensity and duration of the chemiluminescent reaction are enhanced.

Western blots were incubated in blocking buffer at 4°C overnight. All subsequent steps were carried out at room tempertaure on a shaking table (40 rpm) unless stated otherwise. The membranes were washed briefly three times in 100mls of washing buffer prior to one wash of 15 minutes and two washes of 5 minutes in fresh washing buffer. The blots were incubated for 1

hour in primary antiserum diluted 1:1000 in blocking buffer. The washing steps above were repeated before incubation in the secondary serum (peroxidase-conjugated goat anti-rabbit) diluted 1:2000 in blocking buffer. Unbound secondary serum was removed by washing as before but with two additional 5 minute washes in fresh washing buffer. The detection reagents (3mls of each) were combined and poured onto the drained membranes. The luminescence reaction was allowed to proceed for 1 minute before autoradiography. A series of exposures were taken, starting with 1 minute and increasing or decreasing this time as required.

3.2.4 ELISA analysis

96-well plates were coated overnight at 4°C with 100µl per well 10µgml^{-1} of antigen diluted in coating buffer. Plates were then washed three times with 200µl per well washing buffer prior to blocking in ELISA blocking buffer for at least one hour at room temperature. Serum dilutions were applied at 100µl per well in washing buffer and incubated for 90 minutes at 37°C. Unbound primary antibody was removed by washing three times as before prior to incubation with secondary antibody (goat anti-rabbit peroxidase conjugate) at 1:1000 dilution in washing buffer for 90 minutes at room temperature. Plates were again washed three times followed by two washes in PBS to remove unbound secondary antibody. Substrate solution (100µl) was applied to each well and after 20 minutes incubation at room temerature the colour reaction was terminated by the addition of 50µl of stop solution to each well. Absorbance was read at 450nm on a plate reader.

3.3 Immunocytochemistry

3.3.1 Haemonchus contortus

3.3.1.1 Whole worm preparations

3.3.1.1.1 Tissue fixation and permeabilisation

Worms were incubated in fix at 4°C for 6-12 hours, washed three times in PBS for 15 minutes each wash, and stored at 4°C in storage buffer. Worms were permeabilised by incubating at 37°C overnight in BME solution, washing briefly in PBS and incubating for 6-20 hours at 37°C on a shaking table in 120 units (FALGPA) ml^{-1} collagenase in collagenase buffer. Worms were washed in PBS before serum incubation.

3.3.1.1.2 Pre-adsorbtion of primary antiserum for negative controls

HGP1 was added to Anti-HGP1 serum dilutions in antibody dilution solution to a final concentration of 20μg ml^{-1} (4μM). The dilutions were incubated at 37°C for 1 hour and used directly in experiments or stored at 4°C.

3.3.1.1.3 Immunocytochemistry

Primary antibody solutions were prepared in antibody dilution solution. Worms were incubated in these dilutions for 24-72 hours at 4°C. Unbound primary antibody was removed with three 15 minute washes in PBS/Triton. Secondary serum was diluted 1:200 in antibody dilution solution. Incubation was overnight at 4°C, unbound secondary serum was removed by 3 x 5 minute washes in PBS/Triton followed by a long wash overnight. Worms were mounted in "Citifluor™" or mounting medium depending on the fluorochrome used.

3.3.1.2 Cryostat sections

3.3.1.2.1 Tissue fixation and cryostat sectioning

Fixed worms were washed briefly in PBS and incubated at 4°C overnight in cryoprotection solution. Cryoprotected worms were mounted in tissue-tek and frozen on dry ice. 20μm transverse sections were cut along the length of the worm and mounted on poly-L-Lysine coated slides. Slides were used immediately for immunocytochemistry.

3.3.1.2.2 Immunocytochemistry

Slides were incubated at 4°C overnight in dilutions of anti-HGP1 in antibody dilution solution. Unbound primary antibody was removed with three 5 minute washes in PBS/Triton. FITC conjugated goat-anti-rabbit was used as the secondary serum at a dilution of 1:200 in antibody dilution buffer. Incubation was at room temperature for 4 hours. Unbound secondary solution was removed with three 5 minute washes in PBS/Triton as before, and the slide mounted using "Citifluor™" proprietary mounting medium.

3.3.2 Ascaris suum

3.3.2.1 Tissue preparation

Live *A. suum* were maintained in artificial perienteric fluid before injection with 400μl of 4$mgml^{-1}$ collagenase in PBS. Injected worms were incubated at 37°C for 90 minutes and dissected.

Muscle cells were washed away from the body wall with a pasteur pipette, both the muscle cell preparation and body wall were fixed for 6-12 hours at 4°C in PFA fixative. Fixed tissue was washed 3X in PBS prior to storage at 4°C in storage buffer.

3.3.2.2 Immunocytochemistry

Cells were washed in PBS then incubated at 4°C in dilutions of anti-HGP1 in antibody dilution buffer for 12-40 hours. The unbound primary antibody was removed with three 15 minute washes in PBS/Triton. Secondary incubation was for 5 hours at room temperature in a 1:200 dilution of rhodamine labelled goat-anti-rabbit in antibody dilution buffer. The cells were washed three times for 15 minutes in PBS/Triton. Cells were mounted in mounting medium and viewed under Leica DMRB and BiodRad confocal microscopes.

3.4 Cloning of the 5'end of the Hg2/3 cDNA

3.4.1 RNA extraction

RNA extraction was performed using a modified form of the guanidinium thiocyanate/phenol/chloroform method (Chomzcynski and Sacchi 1987). 1g of tissue was frozen and ground to powder under liquid nitrogen. The powder was dissolved rapidly and homogenised in 10 mls of denaturing solution . Subsequently, 1ml of 2M NaOAc pH4.0, 10ml of water saturated phenol and 2ml of chloroform/isoamyl alchohol (49:1) were added in succession with mixing. The solution was chilled on ice for 15minutes before centrifugation at 10,000g for 20 minutes. The supernatant was added to 10mls of denaturing solution and precipitated with 3mls of 2-isopropanol for at least two hours at -20°C. After another centrifugation at 10,000g for 20 minutes the RNA pellet was resuspended in 3mls of denaturing solution and precipitated again at -20^{o}C for at least one hour. The pellet was washed with 75% ethanol, dried and dissolved in 0.5mls of 0.5% SDS.

3.4.2 mRNA Purification

Messenger RNA was purified using "Dynabeads™", polystyrene beads approximately 2.8μm in diameter which have a ferrous core and 25-mer Poly (T) oligonucleotides covalently bound to their surface. 200μg of RNA in 100μl of DEPC-treated water was denatured at 65°C for 10 minutes. Meanwhile, 0.2mls of Dynabead suspension was aliquoted into a 1.5ml microfuge tube and the beads separated from the solution using the magnet supplied. After 30 seconds the supernatant was removed and discarded, and the beads washed in 200μl of 2X binding buffer.

The magnet was removed and the beads resuspended in 100µl of 2X binding buffer. The RNA was then added to the beads with gentle mixing and the mixture allowed to hybridise for 5 minutes on the bench. The tube was put back on the magnet and the supernatant removed and discarded. The beads were washed twice with 200µl of washing buffer before resuspension in 2mM EDTA pH7.5. The solution was heated to 65°C for 2 minutes before separation on the magnet. The supernatant was used immediately for cDNA synthesis or frozen at -70°C.

3.4.3 cDNA Synthesis

The 30µl of mRNA solution was added to 10µl of 5x first strand buffer, 1µl of RoRi-dT linker primer ($1\mu g\mu l^{-1}$), 2µl of 4xdNTPs (10mM), 1µl RNAsin and 2µl of Superscript™ reverse transcriptase. The volume was made up to 50µl and the reaction allowed to proceed for 1 hour at $37^{o}C$. A sample (5µl) of the reaction mixture was removed for a radioactive monitoring and added to 1µl of [$\alpha^{32}P$] dCTP. This was also incubated for 1 hour at 37°C.

3.4.4 Removal of unincorporated $\alpha^{32}P$ [ATP] from labelled DNA samples

A 1ml syringe was plugged with siliconised glass wool and Sephadex™ G25 added to give a bed volume of 1ml. The column was centrifuged at 2000 rpm in a benchtop centrifuge before loading 20µl of STE buffer to equilibrate. The column was centrifuged as above before use. 4µl of 5xSTE and 10µl of water were added to the 6µl of radioactive reaction mixture. This mixture was loaded onto the column which was then centrifuged as before.

3.4.5 Alkaline agarose gel electrophoresis.

3.4.5.1 Preparation of radioactive markers

Phage λ DNA cut with HindIII restriction enzyme was labelled with [$\gamma^{32}P$] ATP. 1µg of digested phage DNA was incubated at 37°C for 1 hour with 1µl [$\gamma^{32}P$] ATP, 1µl T4 polynucleotide kinase ($1unit\ \mu l^{-1}$) and 1µl of 10x PNK buffer in a total volume of 10µl. Unincorporated nucleotides were removed as above.

3.4.5.2 Preparation of 0.8% alkaline agarose gels.

Electrophoresis grade agarose (0.8g) was added to 72mls of water and dissolved by heating in a microwave oven. The solution was allowed to cool to 55°C before addition of 8mls of 10X alkaline electrophoresis buffer. The gels were poured as above except that only 65mls was used

to produce a thin gel. The gels were allowed to set before placing in a gel tank containing 1x alkaline electrophoresis buffer. An equal volume of 2x alkaline gel loading buffer was added to the samples prior to loading onto gels which were run for 7 hours at $600mVcm^{-1}$. Gels were dried down and autoradiographed.

3.4.6 The Polymerase Chain Reaction.

Amplification of inhibitory amino acid receptor subunit cDNA fragments was performed using the PCR. The reactions were set up as shown below.

10µl	Reaction Buffer (Promega)
5µl	4X dNTPs (4mM)
5µl	Primer 1 (20µM)
5µl	Primer 2 (20µM)
2-8µl	$MgCl_2$ 25mM
1µl	cDNA template
Water to 99µl	

A range of magnesium concentrations was used, typically 1,2,3 and 4 mM, for each reaction to give the best yield of specific products. The reaction mix was overlayed with sterile mineral oil in a 0.5ml microfuge tube. The tube was heated to 95°C for 5 minutes to destroy any secondary structure in the cDNA. *Taq* DNA polymerase (0.5µl) was added and the reaction mixture was thermally cycled. The exact cycling conditions used in the PCR varied according to the predicted T_ms of the two primers in use, typically 35 cycles of

Denature	30sec	95C
Annealling	1min	55C
Extension	1min30sec	72C
then..		
Final extension	7min	72C

3.4.7 Screening of PCR Products

3.4.7.1 Screening of PCR products by Southern blotting

The PCR products were run out on a 1% agarose gel which was photographed with a ruler adjacent to it to aid orientation and analysis of the final blot, and immersed in denaturation solution with gentle agitation for 30 minutes. This was repeated giving a total incubation of 1 hour. The denaturation solution was discarded and replaced with neutralisation solution and again incubated for 30 minutes with gentle agitation. This step was repeated once. A piece of Hybond-N membrane was cut to the same size as the gel and placed on the gel, which was then transferred to a Southern blot apparatus (Sambrook *et al.* 1989). Care was taken to ensure that no air bubbles were trapped between the gel and membrane. The gel was surrounded in cling film to prevent the SSC wicking past the gel straight into the absorbent material above. The blot was left for 4-16 hours after which the membrane was removed and washed briefly in 2xSSC. The membrane was allowed to air dry before the DNA was covalently bound to the membrane by irradiating for 5 minutes, DNA side down, on a UV transilluminator.

3.4.7.1.1 Oligonucleotide labelling

Oligonucleotides were labelled with [γ^{32}P]ATP, using polynucleotide kinase (PNK). The following reaction was set up.

2µl	20µM oligonucleotide
5µl	[γ-^{32}P] ATP (2mCi/ml)
1.5µl	PNK (10 units μl^{-1})
1µl	PNK buffer

The volume was made up to 10µl with H_2O and the reaction incubated for 60 minutes at 37°C.

3.4.7.1.2 Hybridisation of Membranes

The membrane was prehybridised with shaking at the hybridisation temperature for at least one hour in 30 mls of prehybridisation solution . The oligonucleotide probe (40pmols) was added and hybridisation allowed to proceed for 4 hours. The filters were washed for 15 minutes in wash solution at the hybridisation temperature, then for 30 minutes at the same temperature in fresh wash solution. The final high stringency wash was carried out in wash solution at 5°C above the hybridisation temperature. During the final wash the blot was monitored with a Geiger counter every minute and removed when the background level had dropped below 25cps. The membrane was air dried and autoradiographed overnight.

3.4.7.2 Screening of PCR Products Using the "Sequenase™" PCR product sequencing kit.

The band of interest was purified using the Sephaband kit and resuspended in 9µl of water, 1.5µl of 10x *Taq* buffer, 2µl of exonuclease I and 2µl of shrimp alkaline phosphatase. The reaction mixture was incubated at 37°C for 15 minutes, after which the enzymes were inactivated by heating to 80°C for 15 minutes. After cooling on ice, an aliquot of approximately 0.5pmoles of the product was made up to 9µl with water and 1µl of sequencing primer (5-10µM) was added. The product was sequenced using the chain termination method (Sanger *et al.* 1977) according to the instructions supplied by the manufacturers with the kit.

3.4.8 Blunt-ended subcloning of PCR products into pBluescript

The purified PCR product was simultaneously blunt-ended and phosphorylated. PCR products generated using *Taq* DNA polymerase have a 5' A overhang due to the terminal transferase activity of this enzyme. A 15µl sample of the purified PCR product was added to 2µl of One-Phor-All buffer plus (Pharmacia), 1µl of DNA polymerase I, 1 µl polynucleotide kinase and 1µl of 10mM ATP were added and after 3 minutes 1µl of 4xdNTPs(4mM) were introduced. The reaction mixture was incubated at 37°C for 30 minutes, after which the reaction was terminated by the addition of 2µl of EDTA. pBluescript™ was digested with EcoRV and the enzyme subsequently inactivated by a 10 minute incubation at 65°C. The tube was cooled on ice, then the linearised vector was dephosphorylated by adding 6µl of calf intestinal phosphatase (8U/µl) and incubating at 37°C for 20 minutes. The phosphatase was inactivated by adding 5 µl of 0.1M EGTA (pH8.0) and incubating at 65°C for a further 10 minutes. Both insert and vector were run out on a 1% agarose gel, and the quantity of each estimated before a further sephaband purification. Ligation and subsequent sequencing were performed as described above.

3.4.9 Searching of sequence databases

Databases were searched using the specified sequence and GCG program TFASTA (Devereux *et al.* 1984). The TFASTA method compares the specified amino acid sequence with the products of all six reading frames of the query nucleic acid sequence, using the method of Pearson and Lipman (1988).

4. Preparation of polyclonal antiserum against the synthetic peptide epitope HGP1

4.1 Introduction

4.1.1 Strategies available for studying patterns of nematode gene expression

Several methods have been used to map the expression of genes in nematodes, including *in-situ* hybridrisation, reporter gene analysis and immunocytochemistry. As mRNAs encoding nematode TGIC subunits are rare species (Cully *et al.* 1994), and the subunits themselves are only present at low levels, it was important to use a highly sensitive method for this study. Sequence alignments (appendix 1) show that some regions of nematode inhibitory TGIC subunits share high levels of amino acid identity, therefore the localisation technique used had to be sufficiently specific to discriminate between Hg1 and related subunits.

4.1.1.1 Immunocytochemistry

The expression patterns of neuronal proteins from many nematode species, including *H. contortus,* have been studied using immunocytochemistry, (Keating *et al* 1995, Brownlee *et al* 1994). Immunocytochemistry can be defined as the demonstration of antigens in tissues using a specific antibody-antigen reaction coupled to a marker, which may be a fluorochrome, electron dense structure or enzyme (Beesley 1993). The main disadvantage of this technique is that a pure antigen must be derived in order to raise an antiserum and the affinities and titre of the antiserum itself must then be characterised before subsequent experiments. The antigen can be derived by purification from native tissue, expression and purification of a recombinant protein or synthesis of a peptide.

4.1.1.1.1 Whole antigen or synthetic peptide?

Anti-sera against peptide antigens have several advantages over those raised against recombinant or native protein. This method circumvents the need to purify the protein from native tissue or a bacterial culture. Judicious choice of peptide sequence permits the production of antisera which can discriminate between proteins which share regions of considerable identity, an approach which has been used to study $GABA_A$ receptor isoforms in bovine brain (Endo and Olsen 1992). In contrast, antisera raised against whole protein have affinities for many epitopes spread over the

surface of the molecule, some of which may be conserved in related proteins. Such antisera are likely to cross-react with related TGIC subunits.

Peptide antisera suffer from one major drawback - an antiserum with a high titre against the free peptide will not necessarily have a high affinity for the native protein (Beesley 1993). Although the peptide sequence can be chosen to include regions which are likely to be antigenic determinants, it is impossible to predict with complete certainty that the selected region will be at the surface of the native protein and therefore accessible to antibodies, or if this region of the native protein is in a similar conformation to that of the peptide. Several factors have been shown to increase the probability that an antiserum raised against a peptide will recognise the parent protein. The chosen peptide sequence should be as long and hydrophilic as possible and chosen from a region near the C- or N-terminus of the parent protein (Stephenson and Duggan 1990). Antisera raised against sequences which include one or more proline residues often retain a high titre against native protein (Harlow and Lane 1988).

4.1.1.1.2 Monoclonal or polyclonal anti-sera?

Monoclonal antibodies offer some advantages over polyclonal antisera as they are potentially very specific. They are much more difficult to produce however, and monoclonal antibodies raised against peptide antigens are even more likely than polyclonal antisera to have low titres against the native protein. Slight conformational differences between the peptide and its parent protein can cause a significant loss of affinity. Similarly the affinity of a monclonal antibody for antigens in immunocytochemical preparations is strongly influenced by tissue fixation (Catty 1988). If the single epitope is destroyed or masked during fixation all affinity is lost. Contrastingly, polyclonal antisera display a heterogeneous response, recognising a range of determinants spread over the chosen peptide, reducing the effect of such local change. In view of these drawbacks and the effort entailed in their production, monoclonal antibodies were not deemed suitable for these experiments.

4.1.1.1.3 Carrier proteins

Small molecules (<5-10Kd) are ineffective immunogens as they are too small to elicit a significant immune response (Catty 1988). Conjugation of these haptens to a larger immunogen increases the antigenicity of the hapten itself. A suitable carrier protein must not be expressed in the tissue in which the antiserum is to be used for immunocytochemical studies and large enough to elicit helper T-cell responses in the host animal. The most commonly used carrier proteins are

4.1.1.1.4 Conjugation methods

The ideal conjugation method couples the peptide to the carrier protein in a conformation which is representative of that found in the native protein. Those methods which couple via the terminal residues of the peptide have been shown to be most effective at achieving this (Stephenson and Duggan 1990, Endo and Olsen 1992). The glutaraldehyde and carbodiimide (EDAC) methods couple the terminal residues of carrier and peptide, however conjugation also occurs at internal lysine residues, in the case of the glutaraldehyde technique, or at aspartate and glutamate residues with the EDAC method. The *m*-maleimidobenzoic acid *N*-hydroxysuccinamide ester (MBS) and bis-diazo-*o*-tolidine (BDOT) methods conjugate through tyrosine and cysteine residues of the peptide respectively (reviewed in Stephenson and Duggan 1990, Catty 1988). The choice of conjugation method therefore depends heavily on the sequence of the selected peptide; sequences which contain lysine and aspartate or glutamate are unsuited to the first two methods and can be altered by the addition of a unique terminal cysteine or tyrosine residue. This allows terminal conjugation with the MBS or BDOT method.

4.1.1.1.5 Adjuvants

Adjuvants enhance the antibody response to injected antigen in a number of ways. Oil-based agents form an emulsion of antigen-containing droplets on mixing with aqueous conjugate. When injected intramuscularly this stable emulsion forms a depot from which the antigen is released gradually, thereby prolonging and increasing the hosts immune response. Alum-based adjuvants give a similar prolonged release from an antigen/alum preciptate. Other preparations, such as Freund's complete adjuvant (FCA), contain heat-killed mycobacteria which stimulate the immune response at lymph nodes and at locally induced granuloma. The tendancy of FCA to induce granuloma formation and autoimmune reactions has led to its withdrawal. Hunter's titermax™ is an effective substitute, containing a metabolisable non-toxic oil and a synthetic polymer adjuvant, which produce a stable emulsion when mixed with the antigen. Sustained release from intramuscular injection sites produces a strong humoral response without causing suffering to the host animal.

4.1.1.2 In-situ hybridisation

In-situ hybridisation has been used to study patterns of gene expression in *C. elegans* (Gomezsaladin *et al.* 1994, Zhen *et al.* 1996). RNA or DNA probes incorporating nucleotides labelled with a radioactive isotope, fluorescent group or hapten are synthesised by *in vitro* transcription (Epstein and Shakes 1995). These probes are then hybridised to permeabilised

4.1.1.2 In-situ hybridisation

In-situ hybridisation has been used to study patterns of gene expression in *C. elegans* (Gomezsaladin *et al.* 1994, Zhen *et al.* 1996). RNA or DNA probes incorporating nucleotides labelled with a radioactive isotope, fluorescent group or hapten are synthesised by *in vitro* transcription (Epstein and Shakes 1995). These probes are then hybridised to permeabilised whole worms or cryostat sections and the transcription pattern of the target mRNA can be visualised by staining or autoradiography, depending on the labelling protocol used. *In-situ* hybridisation is potentially extremely specific as the stringency of hybridisation can be controlled so that the probe will only anneal to near-identical nucleic acid species, however only the pattern of RNA transcription is revealed, not that of expressed protein. TGIC subunits and the mRNAs which encode them have been shown to be transported considerable distances along the neuronal cytoskeleton from their site of synthesis in the cell body to their eventual loction at the synapse (Steward and Banker 1992). As a result of this, the patterns of mRNA transcription and protein expression may differ significantly at the intracellular level. In any case, the high sensitivity requirement considered earlier would dictate the use of a radioactively labelled RNA probe in combination with autoradiography. The small size of *H. contortus* renders this impractical. To date, no *in-situ* hybridisation data has been published from this species.

4.1.1.3 Reporter gene constructs

Reporter constructs which include the start codon and reading frame of a reporter gene, typically encoding β-galactosidase or green fluorescent protein, have been developed for the stable, non-integrative transformation of *C. elegans* (Fire *et al* 1990). The upstream sequence of the gene of study, containing the promoter and *cis*-acting control elements, is cloned into such a vector which is then microinjected into the ovaries of female *C. elegans* in combination with a vector containing a marker gene. F1 progeny displaying the marker phenotype are cultured and the expression pattern of the reporter gene is assayed by histochemical staining or visualisation under UV light. This method has been used to study the expression pattern of the GluClβ subunit (Laughton *et al.* 1997).

Reporter gene analysis is unsuitable for use in this study for several reasons. As some control elements are also found in distant upstream, intronic and 3' sequences, data from reporter experiments are only suitable for the confirmation of expression patterns derived using other techniques. Transformation techniques and reporter constructs are not yet developed for *H. contortus* and *in vitro* culture techniques for its full life cycle are complicated, requiring the use of gastric secretions and host blood (Stringfellow 1986). *C. elegans* has been proposed as a

suitable expression system for parasitic nematode genes (Kwa *et al.* 1995), however the extent to which control elements are conserved between nematode species is not known. As reporter constructs usually contain a 5' signal sequence which directs expression of the reporter gene to the nucleus of the cell, this method shows which cells express the protein of study, but does not reveal the precise intracellular distribution.

4.2 Results and discussions

4.2.1 Design of the synthetic peptide

The peptide was chosen from the putative extracellular domain close to the N-terminus. In addition to the previously discussed suitability of terminal regions, less tissue permeabilisation is required to allow antibodies access to such antigens during immunocytochemistry than for intracellular epitopes. Two other factors influenced the choice of peptide sequence; the predicted antigenicity of the peptide and the level of identity shared with related subunits.

4.2.1.1 Antigenicity considerations

The Antigenic Index, calculated according to the method of Jameson and Wolf (1988), is a measure of the probability that a given region is antigenic. Measures of hydrophilicity (Hopp and Woods 1981), flexibility (Karplus and Schultz 1985), surface probability (Emini *et al.* 1985) and secondary structure (Garnier *et al.* 1978 and Chou and Fasman 1978) were combined according to the formula

$$AI = 0.3\times[H]+0.2\times([Cs]+[Rs])+0.15\times([S]+[F])$$

Where:
AI=Antigenic Index
[H]=Hydrophilicity (Hopp and Woods)
[Cs]=Secondary Structure (Chou-Fasman)
[Rs]=Secondary Structure (Robson-Garnier)
[S]=Surface Probability (Emini)
[F]=Flexibility (Karplus-Schultz)

suitable expression system for parasitic nematode genes (Kwa *et. al.* 1995), however the extent to which control elements are conserved between nematode species is not known. As reporter constructs usually contain a 5' signal sequence which directs expression of the reporter gene to the nucleus of the cell, this method shows which cells express the protein of study, but does not reveal the precise intracellular distribution.

4.2 Results and discussions

4.2.1 Design of the synthetic peptide

The peptide was chosen from the putative extracellular domain close to the N-terminus. In addition to the previously discussed suitability of terminal regions, less tissue permeabilisation is required to allow antibodies access to such antigens during immunocytochemistry than for intracellular epitopes. Two other factors influenced the choice of peptide sequence; the predicted antigenicity of the peptide and the level of identity shared with related subunits.

4.2.1.1 Antigenicity considerations

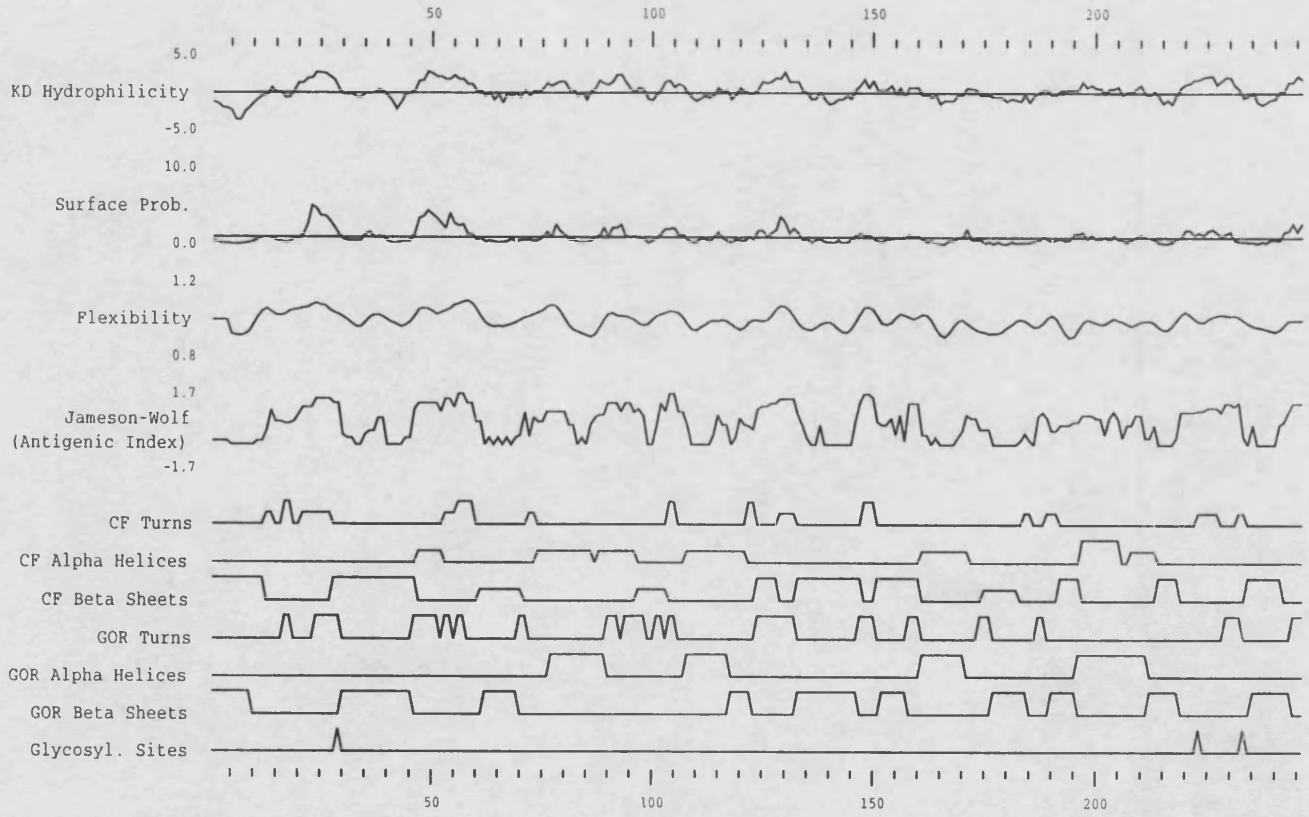
The Antigenic Index, calculated according to the method of Jameson and Wolf (1988), is a measure of the probability that a given region is antigenic. Measures of hydrophilicity (Hopp and Woods 1981), flexibility (Karplus and Schultz 1985), surface probability (Emini *et. al* 1985) and secondary structure (Garnier *et. al.* 1978 and Chou and Fasman 1978) were combined according to the formula

$$AI = 0.3\times[H]+0.2\times([Cs]+[Rs])+ 0.15\times([S]+[F])$$

Where:
AI=Antigenic Index
[H]=Hydrophilicity (Hopp and Woods)
[Cs]=Secondary Structure (Chou-Fasman)
[Rs]=Secondary Structure (Robson-Garnier)
[S]=Surface Probability (Emini)
[F]=Flexibility (Karplus-Schultz)

Figure 12. Antigenicity analysis of Hg1

The predicted N-terminal amino acid sequence of Hg1 was analysed using the GCG program PEPTIDESTRUCTURE.



Structural analysis was performed according to the above equation using the computer program GCG PEPTIDESTRUCTURE. The graphic plot of this analysis (Figure 12) shows several suitable regions with high antigenic indices. The most prominent of these (residues 46-61) was chosen as a candidate region, fortuitously the sequence is proline rich, containing three such residues.

4.2.1.2 Specificity Considerations

A sequence alignment of the selected region was constructed using the GCG program "Pileup" in order to confirm that the domain was sufficiently variable (Figure 13).

Figure 13. Sequence alignment of HGP1 with putative inhibitory "cys-loop" TGICs

The HGP1 sequence is underlined in italics, conserved residues are in bold type.

```
      Hg1   NQDKKFRPTN PDSSPLNVEI
   Cegbr2   DYDWRVRPRG MNATWPDTGG
   Cegbr3   DYDWRVRPRG MNATWPDTGG
   GluClα   GYDFRVRPPT DNGGPVVVSV
   GluClβ   LLDMRVRPPP ANSSTEGAVN
     unc49  TYDKRLRPRY GEKPVDVGIT
  F09C12.1  KLICNEFHDL LQDYDKTMVP
   C53D6.3  DTDIIDRLLN GTGYNKFRIP
   K10D6.1  QPAIIDKLLN GTGYNKFRIP
   F58G6.4  IKFVFRTLTE LKLFFFQHYI
  F55D10.5  DKTKLLNNYK SFRTPSESGV
   F47A4.1  DHLLIDRALY YNKHKLPSPQ
  T20B12.9  LGKLIDTLLT DYDTHLLPEA
   C45B2.4  MQECLKCLIF RHFEEVTVVE
   C09G5.1  MGDVRSAPLR AYAMPSSIGT
  C39B10.2  TQPNIYNLLQ QSNTRPPTAN
   C27H5.8  EQMTVCDLLQ DYDAAVRPSG
   F11H8.2  LIASHDRRIR PNYGGPPIEV
```

An N-terminal cysteine was added during peptide synthesis to allow conjugation of the peptide to thyroglobulin using the (MBS) method, hence the sequence synthesised was CNQDKKFRPTNPDSSPL.

4.2.2 Serum preparation and characterisation

Rabbit no.135 responded strongly to the immunisation and the terminal bleed was carried out after two innoculations. The serum was passed down a thyroglobulin column in order to remove antibodies against thyroglobulin. Antibodies against the carrier protein must be removed by adsorbtion, a process which may reduce the titre against the peptide due to the loss of antibodies against the link region. As epitopes span several amino acids, polyclonal antisera raised against conjugates exhibit affinities for carrier protein epitopes, peptide epitopes and epitopes common to both. The titre of this adsorbed antiserum against the free peptide was assayed using the ELISA to ensure that sufficient affinity remained (Figure 14). This was successful, although the titre against HGP1 was significantly reduced to half-maximal response at 1: 700 dilution. Additionally, the antiserum should show an affinity for a native form of the protein. As purified native protein was not available, the affinity of the antiserum for a recombinant form was assessed. Many prokaryotic expression vectors (Figure 15) are available, in this laboratory repeatable high-level expression of recombinant protein has been achieved using the pMAL vector series (D. Laughton and L. Jamieson, Personal Communication).

4.2.3 Cloning of the extracellular domain of Hg1 into the prokaryotic expression vector pMAL-C2

As the Hg1 cDNA clone did not contain suitable unique restriction sites for directional subcloning into pMAL, a 5' BamHI site and a 3' XbaI site were engineered into the flanking sequences using the PCR. A single band of the expected size of about 700bps was obtained (Figure 16) and subcloned into pMAL-C2. Two transformants were isolated.

Figure 14. ELISA analysis of antiserum titres against thyroglobulin and free HGP1

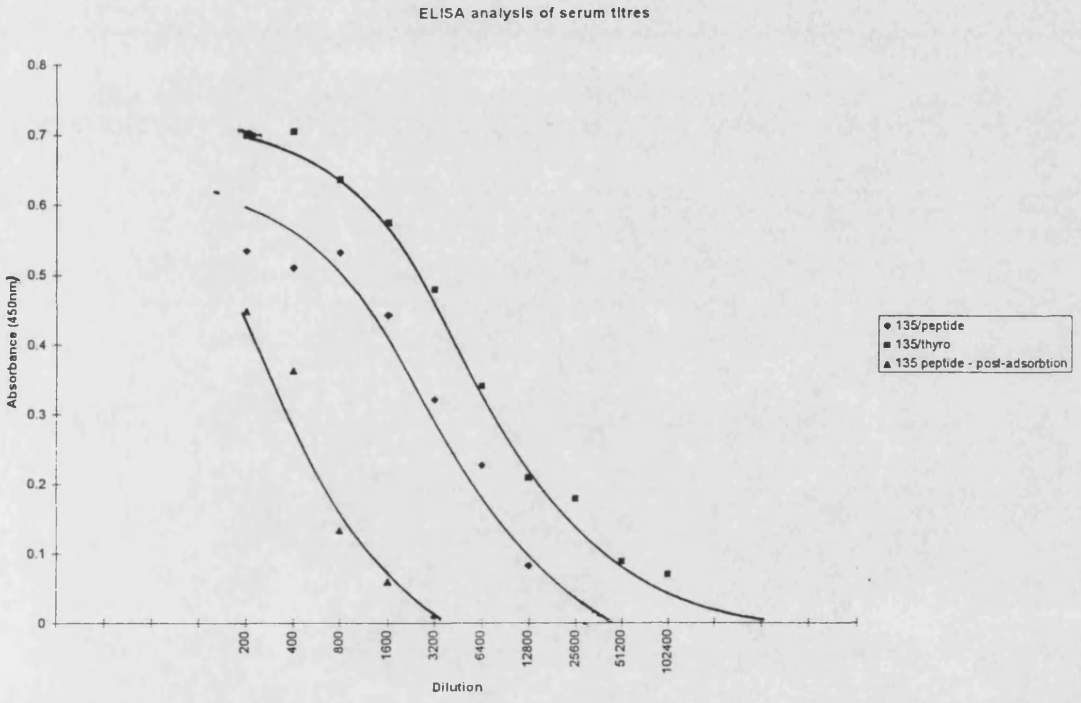
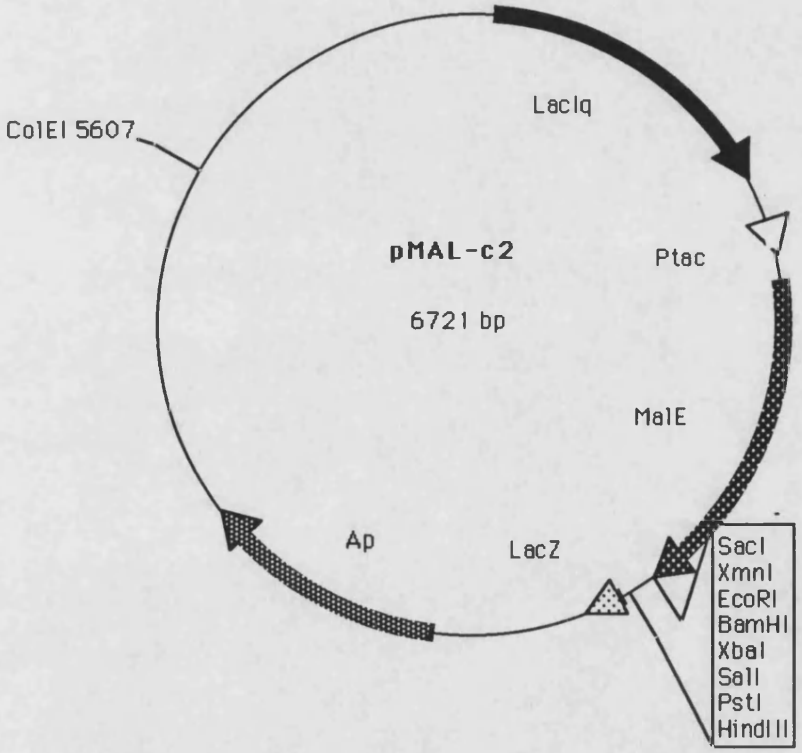


Figure 15. Map of the prokaryotic expression vector pMAL-C2™

The pMAL expression system

The *E.coli* plasmid expression vector pMAL-C2 is a fusion protein expression vector; the peptide encoded by the cloned sequence is expressed as a fusion protein linked by its N-terminus to a 40.6Kd maltose binding protein. Expression of the fusion protein is under the control of the high activity inducible Ptac promoter. Under normal growth conditions the promoter is silent due to binding of the Ptac inhibitor, encoded by the LacIq gene. Addition of IPTG disrupts this interaction resulting in high-level transcription of the Ptac gene product. The malE gene encoding the maltose binding protein (Duplay *et al.* 1984) lies downstream of this promoter, followed by a sequence which codes for a Factor Xa cleavage site. The multiple cloning site is located immediately 3' to this recognition sequence permitting the insertion of a chosen coding sequence in the correct reading frame and subsequent recovery of the protein of study by factor Xa cleavage. Growth of transformed cells in the presence of IPTG induces high-level expression of the maltose binding protein fusion product. pMAL-C2 also includes the LacZ gene to allow blue/white selectionof plasmids containing inserts, and the β-lactamase gene (Ap) which confers ampicillin resistance for the selection of transformants.



4.2.4 Expression of recombinant Hg1 fusion protein

The TB1 strain of E.coli was chosen as a strain suitable for protein expression, as it is protease deficient (appendix 2.). TB1 cells were transformed with 1ng of miniprep DNA from each of the recombinant colonies described above and with uncut pMAL-C2 as a control. SDS-polyacrylamide gel electrophoresis (Figure 18) confirmed that IPTG treatment induced expression of a protein of ~70Kd in each of the recombinant strains and a protein of ~44Kd in the strain transfected with uncut pMAL-C2. The smaller band corresponds to the maltose binding protein encoded by the MalE gene, whereas the two 70Kd products are close to the value of 71Kd predicted from the amino acid sequence. The western blot (Figure 19) shows that anti-HGP1 recognises the fusion protein but not the maltose binding protein alone, confirming that the antiserum is binding specifically to Hg1[ex] domain of the fusion product. The smaller (~48Kd) protein recognised in all cell lysates is an endogenous bacterial protein. This result is not unexpected as the rabbit from which the serum was harvested was likely to have *E.coli* in its gut microflora and therefore may have raised a response to *E.coli* antigens in the recent past.

Figure 1

Figure 16. Gel electrophoresis of Hg1(ex) PCR product

Lane (1)
PCR co

Lane (1) λPst 1 size marker, (2&3) restricted pMAL-C2, (4) Hg1(ex) PCR product. PCR conditions were as described in 3.2.3.1.

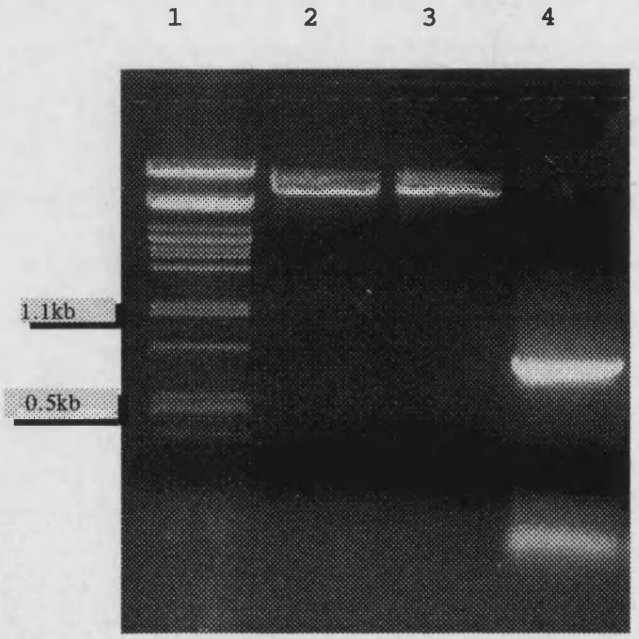


Figure 17. Restriction analysis of plasmid minipreps from E. coli transformed with Hg1(ex) ligated into pMAL-C2.

Lane (1) λPst 1 size marker, (2) uncut pMAL-C2, (3-5 & 7) plasmids containing inserts, (6) re-ligated vector without insert. Uncut plasmid DNA from the minipreps in lanes 3 and 7 was used to transform TB1s for subsequent expression.

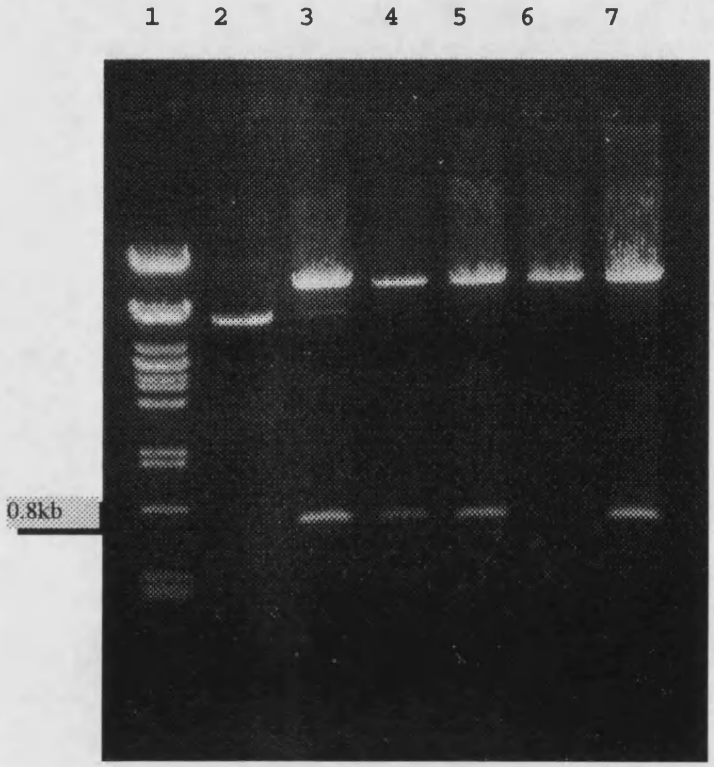


Figure 18. SDS-PAGE of whole cell lysates of cells expressing Hg1(ex).

Lane (1) clone 3 induced, (2) clone 3 uninduced, (3) clone 7 induced, (4) clone 7 uninduced, (5) pMAL-C2 in TB1 induced, (6) Colour markers (Sigma).

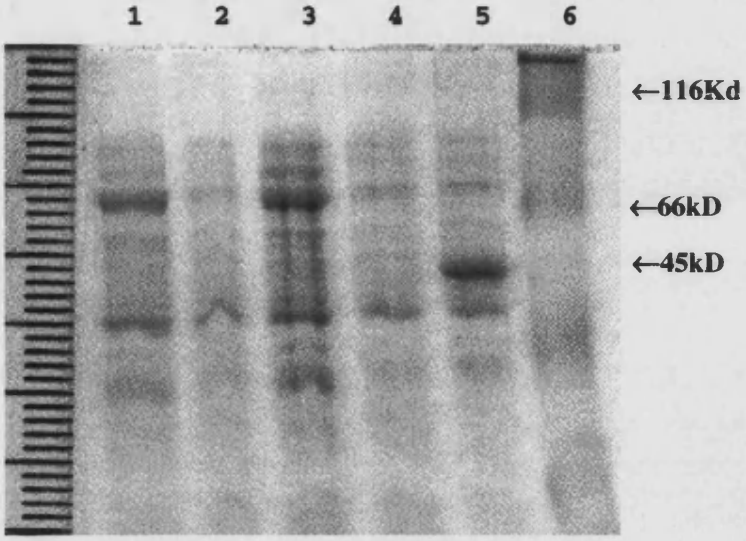
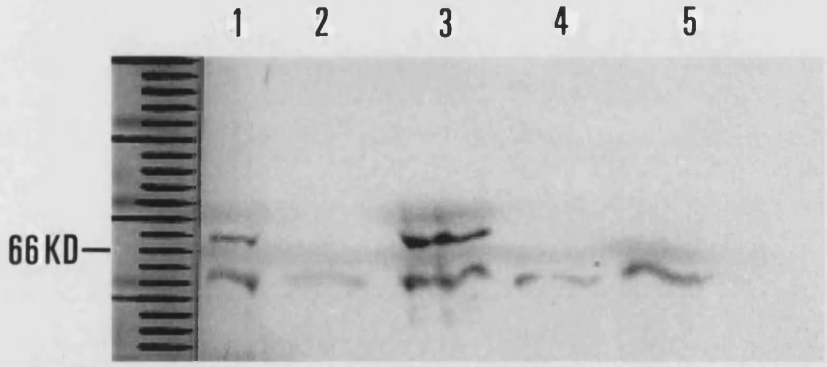


Figure 19. Western blot of SDS-PAGE gel

Lane order as above.



4.3 Conclusions

Anti-HGP1 recognises HGP1 in an ELISA assay with a half maximal response at 1:700 dilution. No significant affinity for thyroglobulin remained after solid-phase adsorbtion. The antiserum also binds specifically to Hg1[ex] fusion protein on a Western blot, at a dilution of 1:1000.

None of the experimental data indicate that the anti-HGP1 serum is unsuitable for use in immunocytochemical experiments, although it is not known if the HGP1 domain of Hg1 is accessible to antibodies when assembled into a functional receptor *in vivo*, or if this domain is found in a similar conformation to the coupled peptide.

5. Immunocytochemical localisation of anti-HGP1 immunoreactivity in *H. contortus* and *A. suum*

5.1 Introduction

Although *H. contortus* is the main organism studied for this project, there is little published work on the neuroanatomy of this species or its use in immunocytochemistry (Veglia 1915, Keating 1995). In contrast, techniques for the processing of *A. suum* tissue are available and its neuroanatomy is well documented. The relatively large size of individual neurons makes their identification more straightforward. For these reasons, both species were used in this study.

5.1.1 *Ascaris suum* - a versatile organism for immunocytochemical studies

The cloning in this laboratory of a number of orthologous subunits from several nematode species and the high level of identity shared by the extracellular domains of orthologues (see chapter 6), suggested that anti-HGP1 might cross react with an Hg1-like subunit in *A. suum*. The large size of this nematode makes it amenable to dissection; Johnson and Stretton (1987) developed a method for the separation of the body wall and associated nerve tracts from muscle cells and gut tissue using *in vivo* collagenase injection. Digested worms are cut open lengthwise and the separated muscle cells recovered by rinsing, leaving a strip of body wall with the attached nervous system exposed. The muscle cells and strip preparations can then be processed independently.

Tissue prepared in this way is ideal for immunocytochemistry, as the surfaces of both neurons and muscle cells are exposed and therefore come into direct contact with antisera during IC, so no further permeabilisation is required. The corollary of this is that antiserum incubation times are likely to be shorter. As the collagenase digestion physically separates muscle arms from the ventral and dorsal nerve cords, it should be straightforward to differentiate between pre- and post-synaptic membranes of the NMJ. Finally, as the gut is removed during the preparation the level of background autofluorescence is reduced. For these reasons the technique has been widely used to study the distribution of classical neurotransmitters and neuropeptides in *A. suum* (Johnson and Stretton 1987, Sithigorngul *et al.* 1991).

5.1.2 The design of immunocytochemistry (IC) experiments

Several factors must be taken into consideration in the design of IC experiments. The tissue sample must be fixed to preserve both tissue morphology and the conformation of the target antigen during prolonged serum incubations and intervening washing steps. Fixed tissues may require permeabilisation to allow antibodies to reach the antigenic site. As polyclonal antisera have a wide range of undefined affinities for other antigens, control experiments must be conducted to determine the specificity of any staining seen. The type of microscopy to be used can influence the choice of both tissue preparation protocol and marker molecule, for example a visible histochemical stain is useless for electron microscopy, whilst samples prepared for optical microscopy are not suitable for viewing under an electron microscope.

5.1.3 Fixation

Tissue samples are fixed to preserve the morphology of the sample and to prevent gradual degradation of the antigen by enzymatic and physical breakdown during IC. Prolonged fixation can result in a loss of antigen/antibody binding as antigenic sites become denatured, or occluded by cross-linking. Therefore fixation is a compromise between the preservation of morphology and the destruction of antigenic sites, the higher the degree of structural resolution required the more stringent the fixation protocol will be and the greater the likelihood of antigen damage. This compromise becomes particularly acute when using electron microscopy.

Aldehyde fixatives are cross-linking agents which give good retention of morphology for optical microscopy with little risk of over fixation. 3-4% Paraformaldehyde (PFA) in phosphate buffered saline (PBS) is the routine fixative for IC on parasitic nematodes (Keating *et al.* 1995, Maule *et al.* 1994, Sithigorngul *et al.* 1991), however membrane-bound epitopes have been demonstrated effectively in glutaraldehyde/PFA fixed tissues (Catty 1988). Although this is a harsher cross-linking agent than paraformaldehyde and can cause antigen damage by overfixation, sufficient antigenic activity survives for effective demonstration of most antigens with polyclonal antisera (Beesley 1993, McIntire *et al.* 1992). Both fixatives were used in this study.

5.1.4 Tissue permeabilisation techniques.

The surfaces of parasitic nematodes perform a variety of vital physiological functions, including evasion of the host immune response (Maizels *et al.* 1993). The predominant feature of a typical nematode body wall is the cuticle, an elastic and resilient layer composed of multiple collagen-rich lipid membranes originally secreted by the hypodermis. Nematode collagens are formed

from pre-procollagens of approximately 30Kd which assemble into mature collagens through the formation of reduction resistant covalent bonds. Mature collagens of 60-120Kd are cross-linked by disulphide bridges which are sensitive to reduction (Cox 1992). The intact cuticle is impermeable to antibodies and hence the integrity of the body wall must be compromised for successful immunocytochemical demonstration of interior antigens. This can be achieved by physical means or enzymatic degradation of the collagen matrix.

5.1.4.1 Mechanical techniques for permeabilising worms

The simplest way to ensure antibody access to the whole worm is to cut the worm into small lengths prior to IC. Unfortunately this method results in disruption of the anatomy near the incision and the permeability of the worm fragment is uneven as antibodies can only reach the regions furthest from the incision by passive diffusion along the body. A more sophisticated extension of this approach is to cut the worm into thin transverse sections using a microtome or cryostat: as the surface of the section is in direct contact with both primary and secondary antisera during IC there are no problems of uneven antibody access. Fixed and embedded adult *H. contortus* were sectioned and used in this study.

5.1.4.2 Enzymatic techniques for permeabilising worms

The cuticle is susceptible to digestion with collagenase after preliminary reduction to cleave crosslinkages. The advantages of this method are that it preserves interior morphology and ensures uniform permeability. The approach is potentially less damaging to antigen potency than embedding or freezing techniques and has been used for IC with *C. elegans* (Laughton 1993, McIntire *et al.* 1992*)*. Collagenase permeabilised *H. contortus* were used in parallel with the cryostat sections.

5.1.5 Controls and blocking agents

The heterogeneous and undefined nature of polyclonal antisera makes the choice of controls in IC experiments critical. Both negative and positive controls should be included in the experiment design. The positive control is usually an antiserum against another epitope known to exist in the organism to be studied, whereas pre-adsorbed or pre-immune antiserum are the usual negative controls. Pre-adsorbed antiserum controls have several advantages over pre-immune. Contamination of the innoculant or infection of the host with microorganisms during the innoculation period may induce an immune response, the antibodies produced as part of this response may give rise to staining of experimental samples which could be misinterpreted as

specific. Consequently, pre-adsorbed serum was chosen as the negative control for this work. Polyclonal rabbit anti-FMRFamide (Peninsula laboratories) was used as the positive control as FMRFamide related peptides have been demonstrated in *H. contortus* neurons using IC (Keating *et al.* 1995).

The addition of blocking agents such as BSA and unconjugated secondary serum significantly reduces background staining due to non-specific binding of both primary and secondary antisera. Surfactants such as Tween and Triton improve tissue permeability and further reduce non-specific background. BSA was used as a blocking agent and Triton X-100 as a surfactant.

5.1.5.1 Direct and indirect immunocytochemistry

For use in direct IC, the primary antiserum is chemically conjugated to a marker and applied to the tissue sample. Unbound antiserum is washed off and the pattern of bound antibodies viewed using a histochemical stain or by visualisation under UV light, depending on the marker selected. The direct approach is quick but insensitive, as there is no signal amplification. Indirect IC, on the other hand, does not require a labelled primary antiserum as a labelled secondary antiserum raised against the immunoglobulin of the primary host animal is used instead. Tissue is incubated in primary antiserum dilutions, unbound antibodies are washed away and the sample incubated with labelled secondary antiserum. Unbound antiserum is washed away once more and the tissue visualised as above. The signal is amplified as several secondary antibody molecules can bind to one primary antibody. Another advantage of this approach is that antisera do not need to be labelled, as conjugated secondary antisera are commercially available. For these reasons indirect IC was used.

5.1.5.2 Conjugated secondary antisera

Affinity purified antisera raised against rabbit IgG are available conjugated to a range of marker groups. The two main classes of marker molecules used for optical microscopy are fluorescent molecules and enzymes. Fluorescent markers emit light when stimulated at their excitation frequency and can be viewed using a confocal microscope. In contrast, enzyme linked antibodies are localised by development in a chromogenic substrate and produce a coloured reaction product which does not fluoresce under UV illumination and is therefore unsuitable for confocal microscopy. The most widely used enzyme conjugates are horseradish peroxidase and alkaline phosphatase. Although inhibitors of endogeonous peroxidase and phosphatase activity are available, high levels of background staining can be encountered when using these enzymes in nematode species (L. Holden-Dye, Personal Communication). Because of this, and to allow the

generation of three dimensional images using a confocal microscope, the fluorescent labels fluoroscein (FITC) and rhodamine (TRITC) were used in this study.

5.2 Results

5.2.1 *Haemonchus contortus*

5.2.1.1 Immunocytochemistry on collagenase permeabilised worms.

Initially samples were fixed with 3% PFA/1% glutaraldehyde and permeabilised with 6 hours of collagenase digestion. FITC-conjugated goat anti-rabbit IgG was used as the secondary serum. Only non-specific staining of the buccal cavity, anus and gut contents were seen; this was due to the secondary serum and was seen in all subsequent experiments with both FITC and TRITC conjugated antisera. When the permeabilisation conditions were increased to an extended (20 hour) reduction step in β-mercaptoethanol (BME) and 9 hours of collagenase digestion, specific HGP1 immunoreactivity was observed along the ventral nerve cord at a primary antiserum dilution of 1:10 (Figure 20, Figure 21). No staining was seen with either the secondary antiserum alone or with pre-adsorbed anti-HGP1 (Figure 22).

The absence of staining at dilutions greater than 1:10 suggests that permeabilisation was incomplete or that the serum titre against native Hg1 was rather low. The background level of fluorescence was high for all dilutions of anti-HGP1 due either to non-specific binding of the secondary serum or autofluorescence. Further experiments using 5% goat serum in the antibody dilution solution showed no significant reduction in background levels, suggesting that it was a result of autofluorescence. To test this, fixed worms were subjected to IC as above but antisera were omitted from all steps. Washed samples were visualised through the FITC and TRITC channels of the microscope and showed considerable autofluorescence at both emission frequencies (Figure 23).

To overcome the low signal to noise ratio, subsequent batches of worms were fixed in 4%PFA in PBS, as glutaraldehyde can cause autofluorescence when used to fix nematode tissue (E. Munn, Personal Communication). In addition to this precaution, samples were imaged using a Biorad confocal microscope when necessary. The selective frequency filters on the confocal microscope and monochroic nature of laser light significantly reduce the level of autofluorescence and therefore increase the signal:noise ratio. TRITC-conjugated secondary serum was then employed, with worms fixed as above. The reduction step in BME solution was maintained at 20 hours and the digestion in collagenase increased to 16 hours. Primary serum was diluted 1:100 and

incubation was for 40 hours at 4°C. Incubation in secondary serum was at room temperature for 5 hours at 1:200 dilution, before an overnight wash at 4°C. This severe permeabilisation resulted in specific staining of two additional structures. The first of these comprised a set of neurons in the region of the nerve ring, which stained in most individuals (Figure 24,Figure 25 & Figure 26). The second HGP1-immunoreactive structure was a single process posterior to the ring which ran around the circumference of the worm from the lateral ganglion, in a ventral direction (Figure 24 & Figure 27). As before, the ventral nerve cord showed HGP1 immunoreactivity (Figure 28). Only non-specific staining of buccal and anal cavities and gut contents was seen in the negative controls.

5.2.1.2 Immunocytochemistry on Cryostat Sections.

Weak but specific staining of muscle membranes opposing the ventral nerve cord was seen at 1:200 dilution of anti-HGP1 (Figure 30). This approach was abandoned in favour of the whole worm method discussed above for a number of reasons. Despite using a range of slide coatings (Poly-L-Lysine, glycerin/chrome alum) most sections failed to survive the many washing steps used during IC. Typically, only the cuticle remained and internal organs were lost. The method also uses large quantities of antiserum, as approximately 700µl of diluted antiserum was required for each slide.

5.2.2 *Ascaris suum*

Specific, particulate staining of muscle arms was seen at primary serum dilutions of up to 1:500 (Figure 31). No staining was seen with pre-adsorbed anti-HGP1, or when the primary serum was omitted altogether (Figure 32 and Figure 33). The immunoreactivity was most pronounced at the very tip of the muscle arm, and limited to its distal half. No staining of the nerve ring and associated commissures was seen in body wall preparations (data not shown).

Figure 20. Male H. contortus stained with anti-HGP1.

Male adult *H. contortus*, ventral view. Anti-HGP1 was used at a dilution of 1:10. Scale bar approximately 50μm.

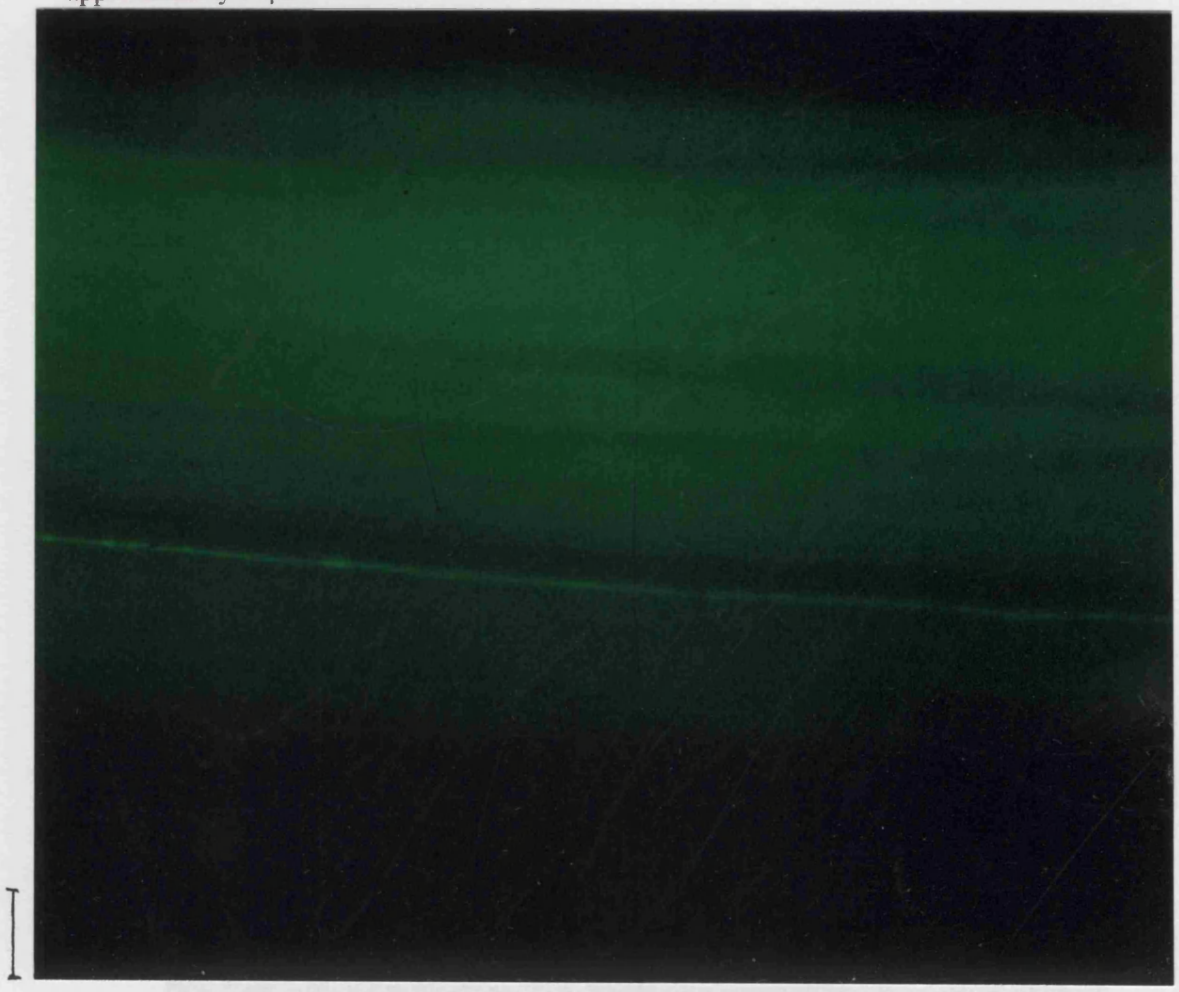


Figure 21. Close up of figure 20.

Scale bar approximately 20μm.

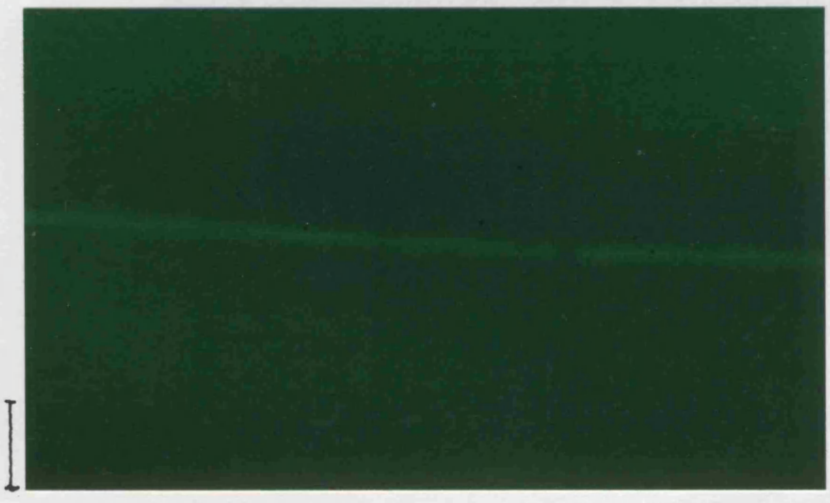


Figure 22. Negative control - female H. contortus stained with pre-adsorbed anti-HGP1.

Adult female, ventral view. Primary serum diluted 1:100 in antibody dilution solution was pre-adsorbed as described in 3.3.1.1.2. Scale bar is approximately 50μm.

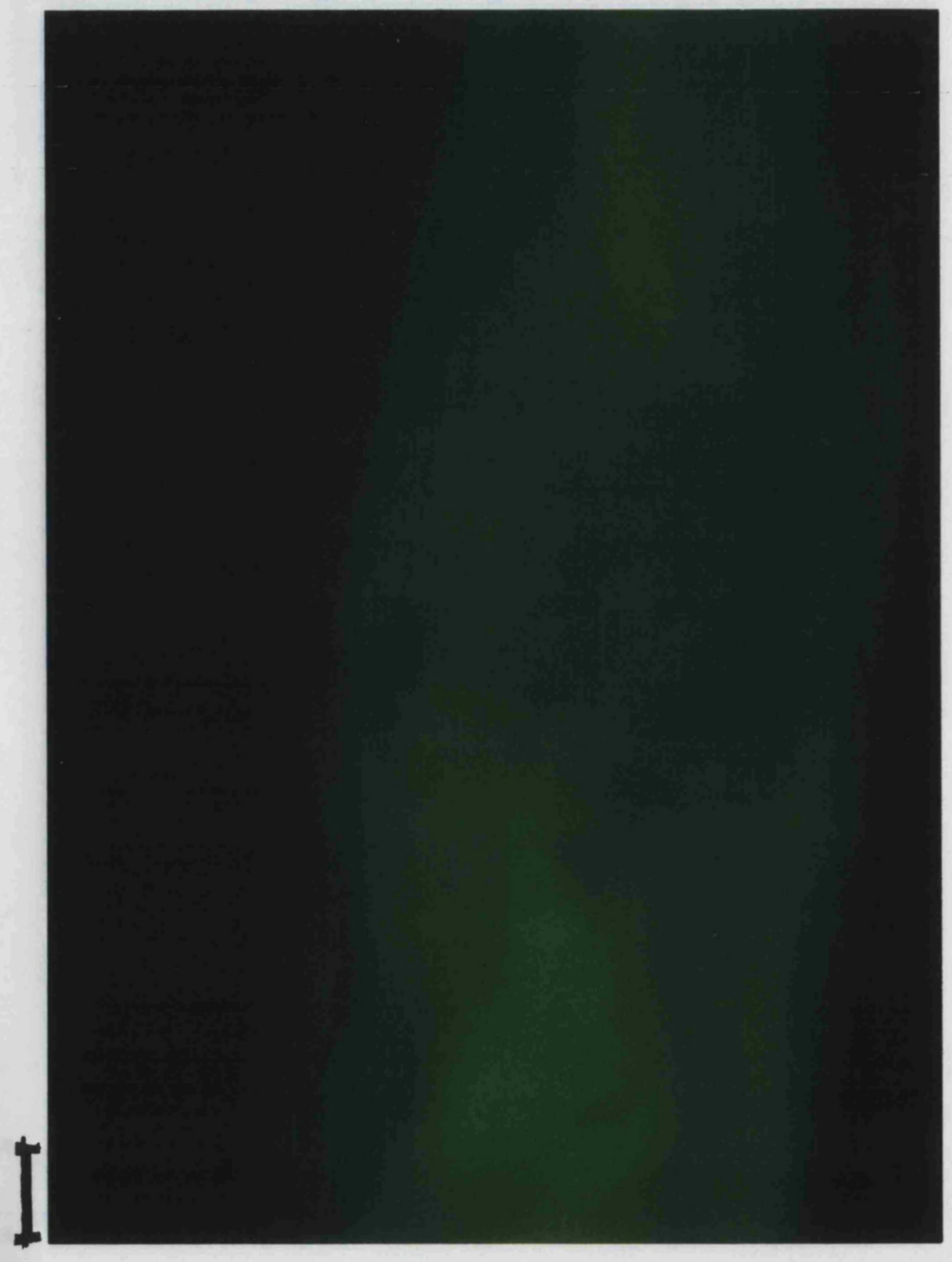


Figure 23. Autofluoresence of fixed H. contortus.

Adult male, dorsal view. The specimen was examined through the FITC (top) and TRITC (below) channels of the microscope. Considerable autofluoresence is visible at both excitation frequencies. Scale bar is approximately 100μm.

Figure 24. HGP1 immunoreactivity in the head of H. contortus.

Adult female, lateral view of head. Anti HGP1 was used at a dilution of 1:100. Scale bar approximately 100µm.

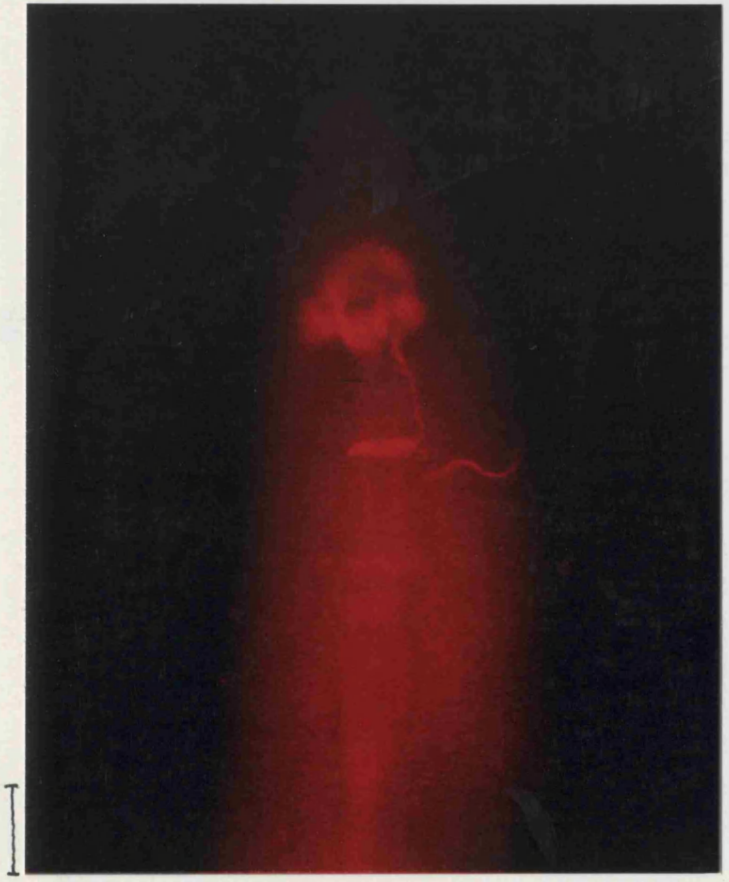


Figure 25. HGP1 immunoreactivity in the nerve ring of H. contortus.

Adult male, ventral view of head. Anti HGP1 was used at a dilution of 1:100, scale bar approximately 100µm.

Figure 26. Confocal Image of the anterior structure from Figure 24.

Ventro-lateral view. Anterior is top right, posterior bottom left. Scale bar approximately 10μm.

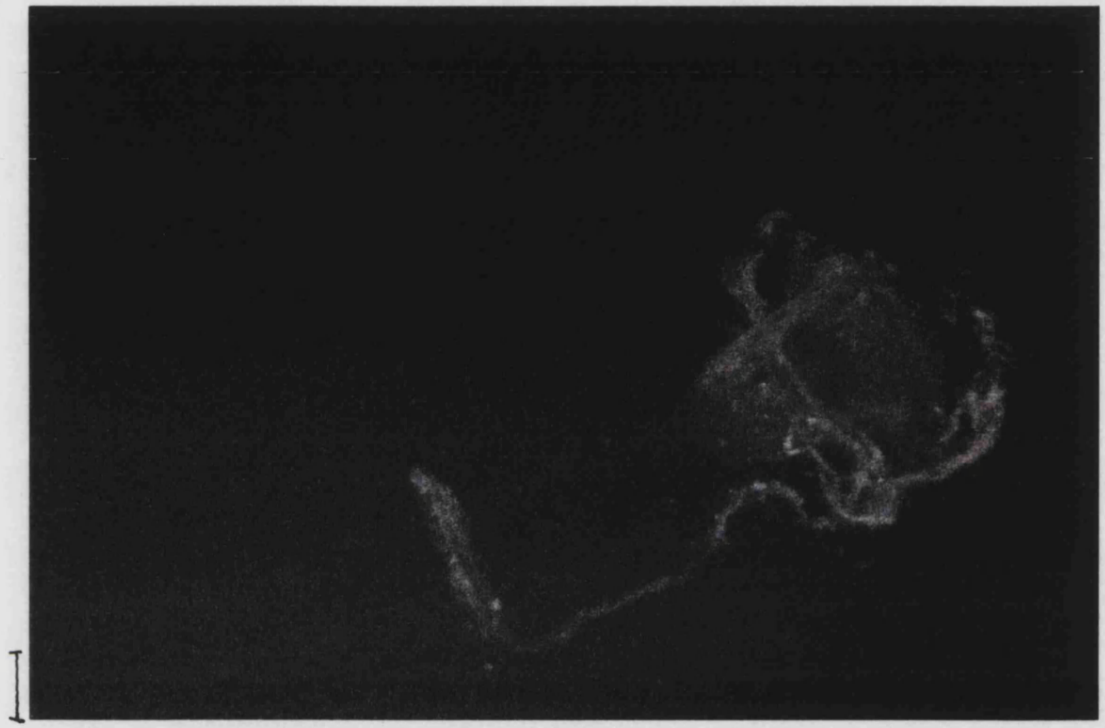


Figure 27. Confocal image of posterior structure from figure 24.

Two confocal series of this immunoreactive cell were generated after the sample had been remounted. The two images were combined manually as a montage. Lateral view (ventral is top left, anterior is top right). Scale bar approximately 20μm.

Figure 28. HGP1-immunoreactive ventral nerve cord - adult female H. contortus.

Lateral view (dorsal is up) showing weak, specific staining of the ventral nerve cord at an anti-HGP1 dilution of 1:100. Scale bar approximately 100μm.

Figure 29. Negative control - adult male H. contortus

Adult male, ventral view. Anti-HGP1 diluted 1:100 in antibody dilution solution was pre-adsorbed with HGP1 as described.

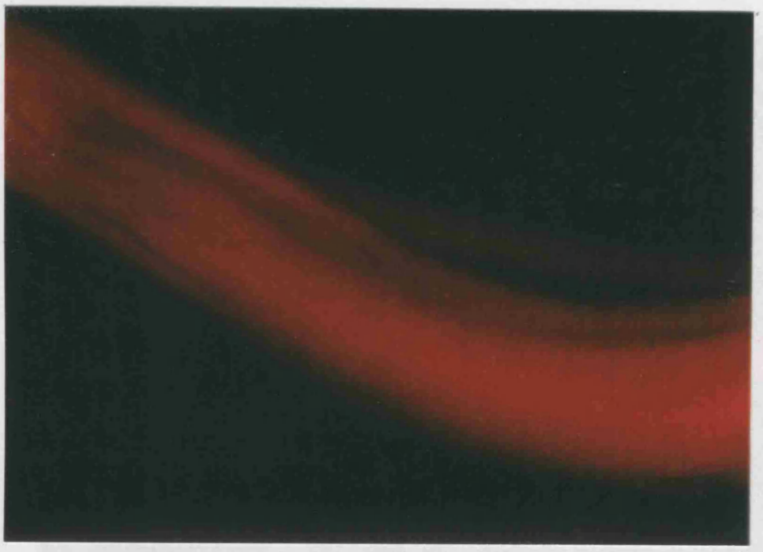


Figure 30. Anti-HGP1 immunoreactivity on tranverse section of adult H. contortus

Transverse section of adult male *H. contortus* stained with anti-HGP1 diluted 1:200.

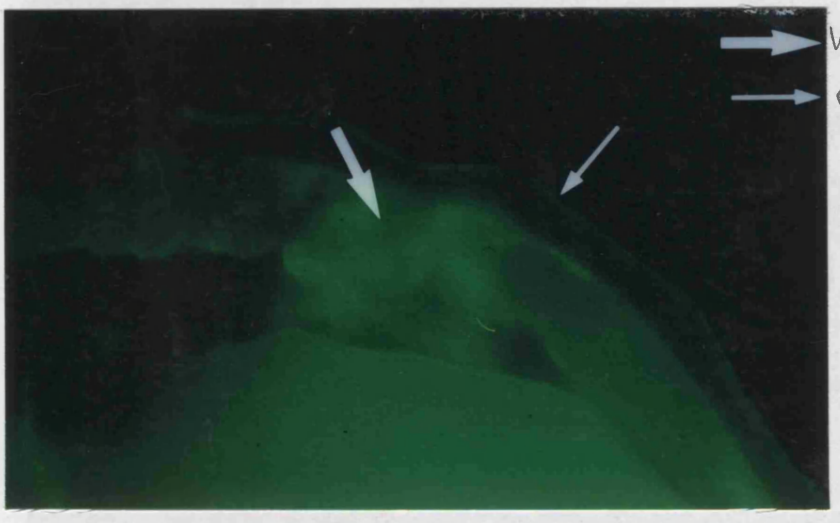


Figure 31. HGP1 immunoreactivity on A. suum muscle arms.

Anti-HGP1 was diluted 1:500. Staining was most pronounced at muscle arm termini. Scale bar approximately 20μm.

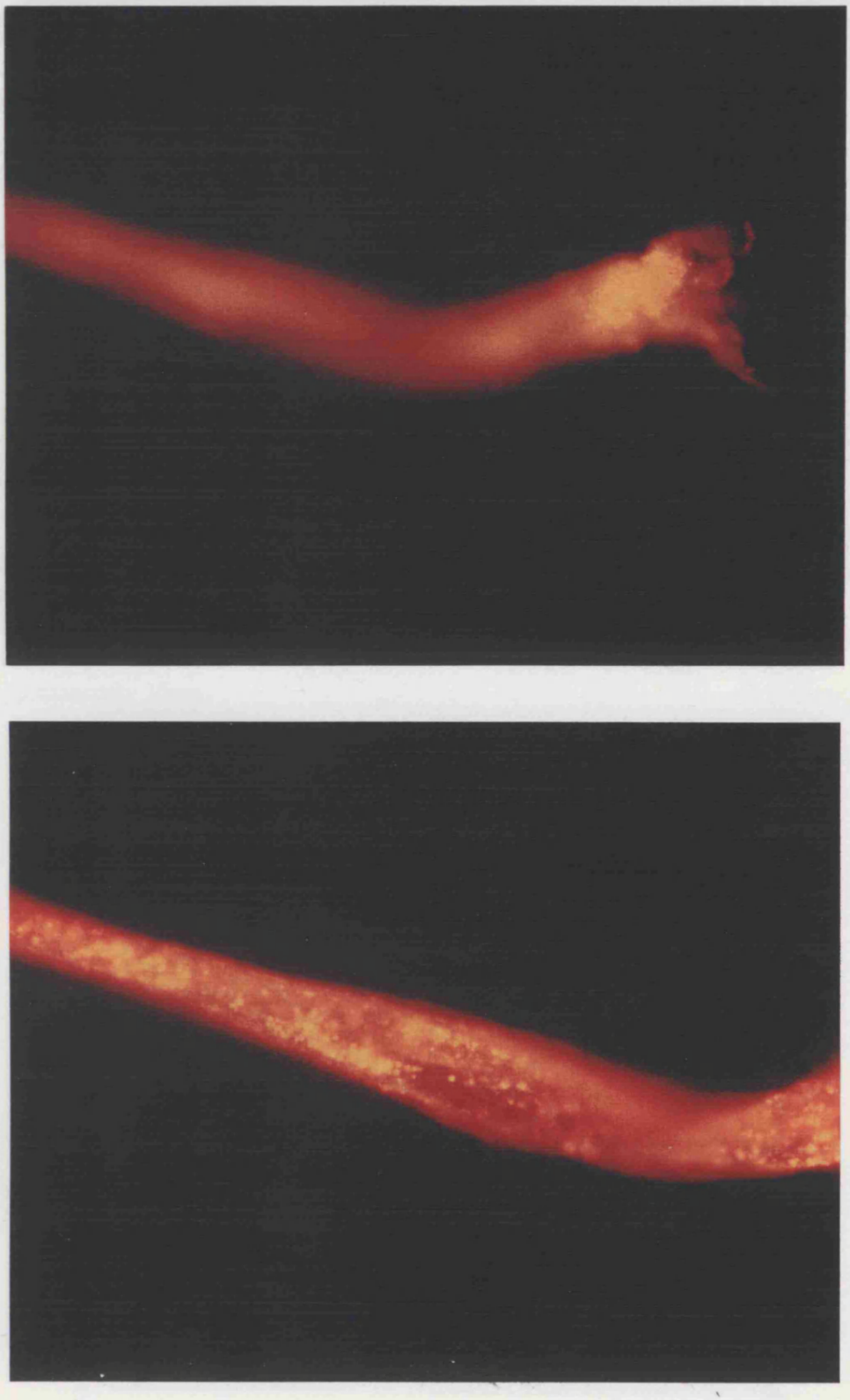


Figure 32. Negative control - omission of primary antiserum

Scale bar approximately 100μm.

Figure 33. Negative control - pre-adsorbed anti-HGP1

Anti-HGP1 was diluted 1:500 and pre-adsorbed as previously described. Scale bar approximately 20μm.

5.3 Discussions

5.3.1 Haemonchus contortus

Taken together, the staining patterns seen in whole worms and sections suggest that HGP1 immunoreactivity is found at the post-synaptic membrane of the neuromuscular junction (NMJ). The nerve cord staining seen with collagenase treated worms is likely to be post-synaptic for two reasons. Firstly, transverse sections show weak but specific HGP1-immunoreactivity at this location (Figure 30). Secondly, the nerve cord staining seen with anti-HGP1 is qualitatively different from that seen with anti-FMRFamide. Worms stained with anti-FMRFamide show a thin line of staining along the nerve cord with a defined boundary, consistent with an intracellular location for FMRFamide. This staining is only visible at high magnification (≥X200 data not shown and Keating *et al.* 1995). In contrast, the pattern of HGP1 immunoreactivity along the nerve cords is slightly more diffuse and wider in diameter.

HGP1 immunoreactivity in the nerve ring is limited to a small subset of the many neurons which have processes which enter this region of neuropile, further evidence of the specificity of the staining. The confocal image of HGP1 immunoreactivity in the nerve ring (Figure 26) was analysed by creating an animated sequence in which the structure is rotated in both the vertical and horizontal axes. From this a representation of the pattern of immunoreactive neurons was drawn (Figure 34)

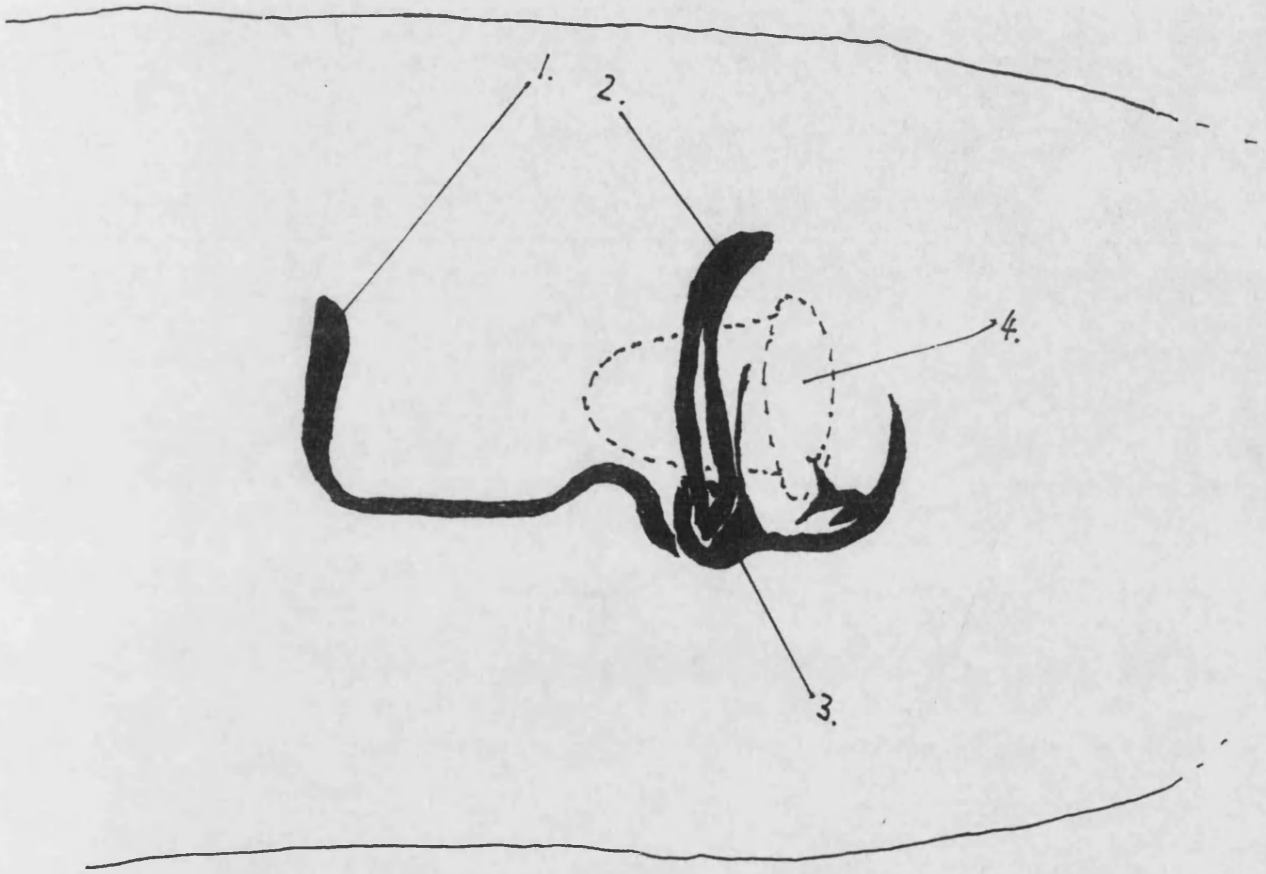
Unlike *A. suum* and *C. elegans*, the neuroanatomy of *H. contortus* has not been studied in any depth. Consequently, information about this species is limited to the anatomical drawings of Veglia (1915). This study was conducted by light microcoscopy of both whole worms and transverse sections from L3 and adult stages and is in close agreement with the published anatomical drawings and photographs from the other two species. Unfortunately, the resolution is limited to the positions and morphology of the major nerve tracts, rather than of individual neurons. The neuroanatomy of *A. suum* is very similar to that of *C. elegans* (Holden-Dye and Walker 1994) and *H. contortus* is phylogenetically much closer to *C. elegans* than *A. suum* is, therefore it may be reasonable to assume that this close agreement extends to the level of individual neurons. Based on this assumption, the neurons in Figure 34 would appear to be members of the RI (ring interneuron) and RM (ring motor neuron) classes described in *C. elegans*. This identification is based on the probable positions of cell bodies and the morphology of neural processes (White *et al.* 1986). The posterior cell (Figure 27), appears similar in structure to the *C. elegans* sensory neuron AQR on the basis of its extensive dendritic processes and the position of its cell body; however the process anatomy differs from that of AQR, lacking

the connection with the nerve ring. Unequivocal identification of this cell was not possible, highlighting the need for further study of the anatomy of this important pathogenic species.

This pattern of HGP1 immunoreactivity at the postsynaptic membrane of body muscle NMJs and on 3-5 RM and RI neurons is consistent with the hypothesis that Hg1 is a GABA receptor subunit. As discussed in section 1.2.3.1.1, GABA is the major inhibitory neurotransmitter at the bodywall NMJ and GABA receptors are found on *A. suum* muscle cells (Martin 1980, 1982). In *C. elegans*, the majority of RM and RI neurons are postsynaptic to RIS and DVB, which are both GABAergic (McIntire *et al.* 1993b).

Figure 34. Diagrammatic interpretation of HGP1 immunoreactive cephalic neurons

The confocal image shown in figure 26 was used to produce a number of animated sequences with the program "confocal assistant". From these sequences the spatial arrangement of HGP1 immunoreactive processes was derived. Ventro-lateral view, anterior is right. The probable positions of cell bodies are shown (1-3), the diffuse pear-shaped immunoreactive structure (4) is the interior of the pharynx. Scale bar approximately 20μm.



5.3.2 *Ascaris suum*

Muscle arm termini form the postsynaptic membrane of the NMJ in live nematodes. The strong anti-HGP1 immunoreactivity observed at this region in *A. suum* is consistent with the pattern of postsynaptic immunoreactivity seen at the NMJ in *H. contortus*. The absence of neuronal staining in *A. suum* may reflect differences in the expression of Hg1 orthologues in the two species, although the small number (4) of flap preparations used in this study makes it inconclusive in this regard. Interestingly, muscle cells from the same individuals were HGP1-immunoreactive.

Differences in GABA distribution in *A. suum* and *C. elegans* have been observed; no staining was seen of an *A. suum* neuron equivalent to the GABEergic RIS cephalic interneuron in *C. elegans* (Guastella *et al.* 1991, Guastella and Stretton 1991, McIntire *et al.* 1993b). *H. contortus* is evolutionarily much closer to *C. elegans* than to *A. suum*, these differences in GABA distribution between species are likely to be reflected in different patterns of GABA receptor subunit expression. If Hg1 is indeed a GABA receptor subunit, this could account for the absence of neuronal HGP1 immunoreactivity in *A. suum*.

The experiments on *A. suum* gave more reproducible staining at lower antibody concentrations than those on whole mount *H. contortus* (1:500-1:1000 for *A. suum* compared to 1:10-1:100 for *H. contortus*). This result is surprising, as the antiserum would be expected to have a higher titre for Hg1 than for a related protein, however there are two explanations. Firstly, the *A. suum* technique gives superior antibody access to both muscular and nervous tissues: permeabilisation was a critical factor in demonstrating HGP1 immunoreactivity in *H. contortus*. Secondly, the larger worm is likely to have a correspondingly greater number of $GABA_A$ receptors on each muscle arm.

In conclusion, HGP1 immunoreactivity was found post-synaptically at the NMJ in both *A. suum* and *H. contortus*, consistent with the hypothesis that this subunit is part of a $GABA_A$ receptor. Between 3 and 5 neurons in the nerve ring and a single cell with its nucleus in the region of the lateral ganglion were HGP1 immunoreactive in *H. contortus*. The cells in the nerve ring were similar to the RI and RM neuron classes in *C. elegans*, the single cell posterior to these was AQR-like. Accurate identification of individual cells was hampered by the lack of detailed information on the anatomy of this species. No staining was observed in *A. suum* neuronal tissue, however only 4 body wall preparations were used in this work so no conclusion can be drawn from this.

Tissue permeabilisation was identified as the most important factor in the design of IC experiments. The Johnson and Stretton method for the preparation of *A. suum* nervous system and muscle cells produces tissue which is ideal for immunocytochemical study, further underlining the utility of this large worm in nematode neuroscience.

6. Cloning of the 5'end of the Hg2/3 cDNA

6.1 Introduction

6.1.1 Cloning techniques

Two methods, library screening and the PCR, are used routinely to clone full-length cDNAs from partial sequences. The advantage of screening a cDNA library is that the full length clone can be isolated in one step and is likely to be free of polymerase induced point mutations which may be present in PCR products. Unfortunately, as TGIC subunit mRNAs are so scarce the library must be made to an extremely high titre and complexity to ensure that TGIC subunit cDNAs are represented. Even using a well-constructed library, millions of plaques may need to be screened before isolating the relevant cDNA clone (Harvey *et.al.* 1991, S. Jagannathan, Personal Communication).

In contrast, PCR techniques are ideally suited to the amplification of rare species from a heterogeneous pool of cDNAs. The relative scarcity of the target cDNA is less critical as few template molecules are required for successful amplification, indeed amplification has been reported from only one template molecule (Holding and Monk 1990). The corollary of this is that less cDNA is required as a template for PCR reactions than for library construction. This method has been used successfully here in Bath to amplify full length and partial TGIC cDNAs from several nematode species (see 1.3.3.1).

6.1.1.1 PCR Methods

In the decade since its discovery, the PCR technique has been adapted for use in a host of applications, several methods have been developed which improve aspects of the reaction's performance (Innis 1990).

6.1.1.1.1 Nested PCR

The specificity of the PCR can be enhanced by using a small fraction of a first reaction as template for a second reaction. The primers used in the first round flank one or more of the second round primers (Figure 35). This method applies a second level of screening to the products from the first reaction, re-amplification can produce a final single product, even if no first round products are visible after agarose gel electrophoresis. As nested PCR strategies usually involve in excess of 60 cycles, the rate of PCR-induced mutation is significant. This

must be taken into account when analysing cloned reaction products and a number of clones should be sequenced in full to give a definitive result.

6.1.1.1.3. Touchdown PCR

The specificity and sensitivity of the PCR can be enhanced by using touchdown techniques (Don *et al.* 1991). Touchdown PCR reactions are set up and run under near-identical conditions to those used for standard PCR, except that the annealling temperature is initially higher than the predicted melting temperature (Tm) of both primers and drops by a fixed amount with each cycle until it reaches a predetermined lower limit, which is maintained for the remaining cycles. For example, if the Tm of both primers is estimated as 57°C, the cycling conditions might start with an annealling temperature of 60°C, falling by 0.5°C each cycle. After twenty cycles the annealling temperature would be 50°C; this temperature would be used for another 30 cycles giving a run of 50 cycles over all. The increased cycle number (50 as compare to ~35 in standard protocols) is required as the primers do not anneal in the early cycles of the reaction and no amplification occurs, however as the temperature falls the primers anneal first to the most closely matching site in the cDNA pool. The initial products are therefore likely to be specific; by the time the annealling temperature has fallen to a level at which non-specific amplification occurs, the cDNA will be enriched for the target cDNA. This stoichiometric "head-start" results in a much more specific amplification than that produced using static annealling temperatures.

6.2 Results

As no gene-specific upstream 5' sequence was known, a hemi-nested PCR strategy was used with SL1 acting as the sense primer in both rounds of amplification (Figure 36). The primers SL1, A0, A1 and A2 (Table 3) were synthesised and deprotected.

6.2.1 Amplification of the ribosomal L9 protein cDNA

6.2.1.1 Template production

Total RNA was extracted from 0.8g of frozen adult *H. contortus*, yielding approximately 550µgs as estimated by agarose gel electrophoresis. mRNA was purified from 200µgs of this sample and used as a template for first-strand cDNA synthesis. To confirm that full-length cDNA species of several kilobases had been transcribed, an aliquot of the reaction was radiolabelled and analysed by alkaline agarose gel electrophoresis and autoradiography (Figure 37).

6.2.1.2 PCR conditions

cDNA (1µl) was used as a template in the first round of the hemi-nested PCR reaction with the primers SL1 and A2. The first PCR reaction used a relatively low (43°C) annealling temperature, whereas the second reaction used a more typical 50°C step with the primers SL1 and A1, to give more specific amplification. Electrophoresis of a fraction of first round products revealed four weak bands with sizes ranging between approximately 500bps and 1kb (Figure 38), whereas the second round reaction produced a single major product of just over 500bps with a background smear of heterogeneous product. The single band was excised from the gel, purified and subcloned into the EcoRV site of the vector pBluescript™. Two of the resulting clones were sequenced (Figure 39).

Figure 36. PCR strategy selected for cloning the 5' end of the Hg2/3 cDNA.

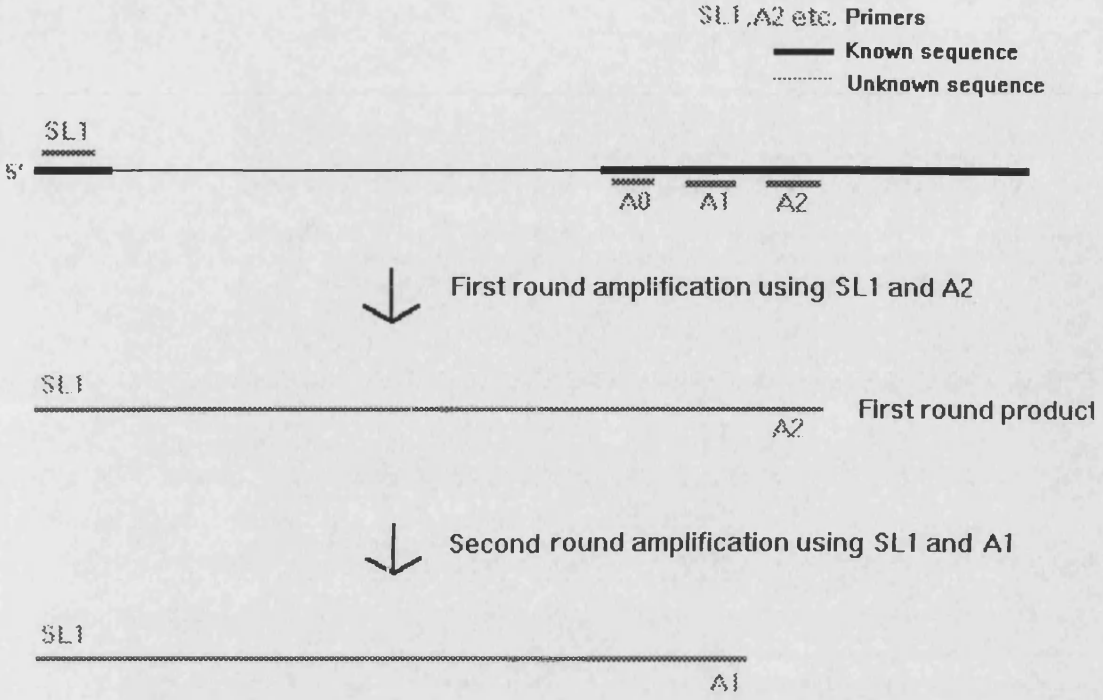


Table 3. Oligonucleotides used for PCR amplifications and screening of PCR products by Southern blotting.

Predicted melting temperatures were estimated according to the method of Itakura *et al.* (1984).

$T_m = 4 X (G/C) + 2 X (A/T)$

where the T_m is in °C, (G/C) is the sum of G and C residues in the oligonucleotide and (A/T) the sum of A and T residues.

Primer	Sequence	Estimated T_m
SL1	GGTTTAATTACCCAAGTTTGAG	60°C
A0	TATGAGATGTCGTCGTGCCTC	64°C
A1	GAGCACGTTCGGCTTGTC	58°C
A2	GGATCGAGCCGTCCTTGTGAA	66°C
DL11	(A/C)T(T/C)TGGGTGCCAGACACCT(T/A)(C/T)TT	66-72°C
DL19	(A/G)(A/G)(A/G)TTNA(A/G/T)CCA(A/G)AAN(C/G)AAN(C/G)(A/T)NA CCCA	68-86°C

Figure 37. Analytical gel of total-RNA extracted from H. contortus and alkaline agarose gel of labelled cDNA synthesis products.

Left: Agarose gel electrophoresis of total RNA. Right: Alkaline agarose gel electrophoresis of labelled cDNA (2), with λHindIII marker (1).

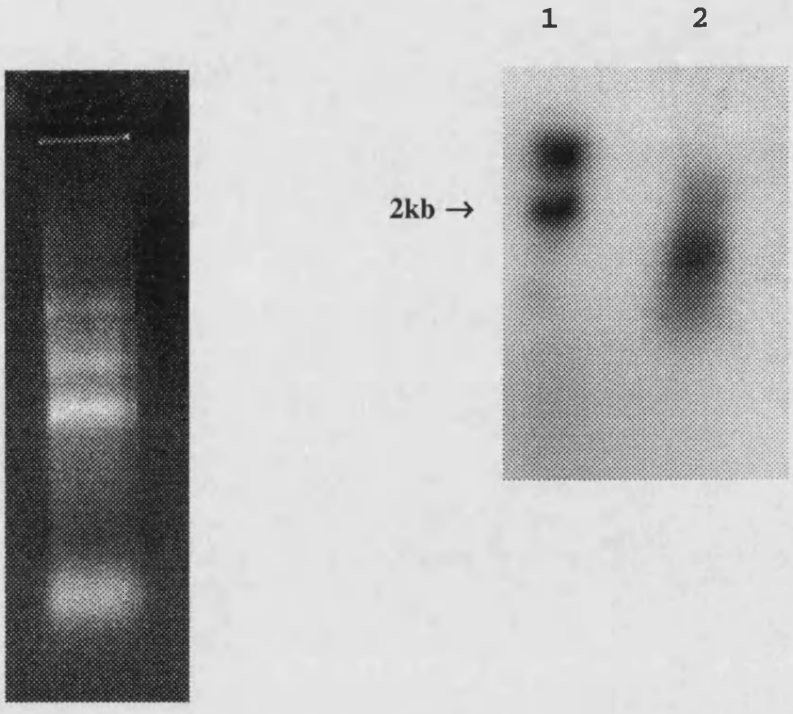


Figure 38. PCR products from 1st and 2nd round nested amplification.

First round - left: After a standard 5 minute heat denaturation, the samples were cycled through 40 cycles of annealling (43°C/90secs), extension (72°C/60secs) and denaturing (95°C/30secs) in the presence of primers SL1 and A2. 2.5units of *Taq* DNA polymerase was used with 1.5mM $MgCl_2$. 25µl of the reaction mixture was electrophoresed through a 1.2% agarose gel.

Second round - right: The reaction conditions were as above except that primer A1 was substituted for A2 and the annealling temperature was raised to 50°C. λHindIII was used as a size marker in both gels.

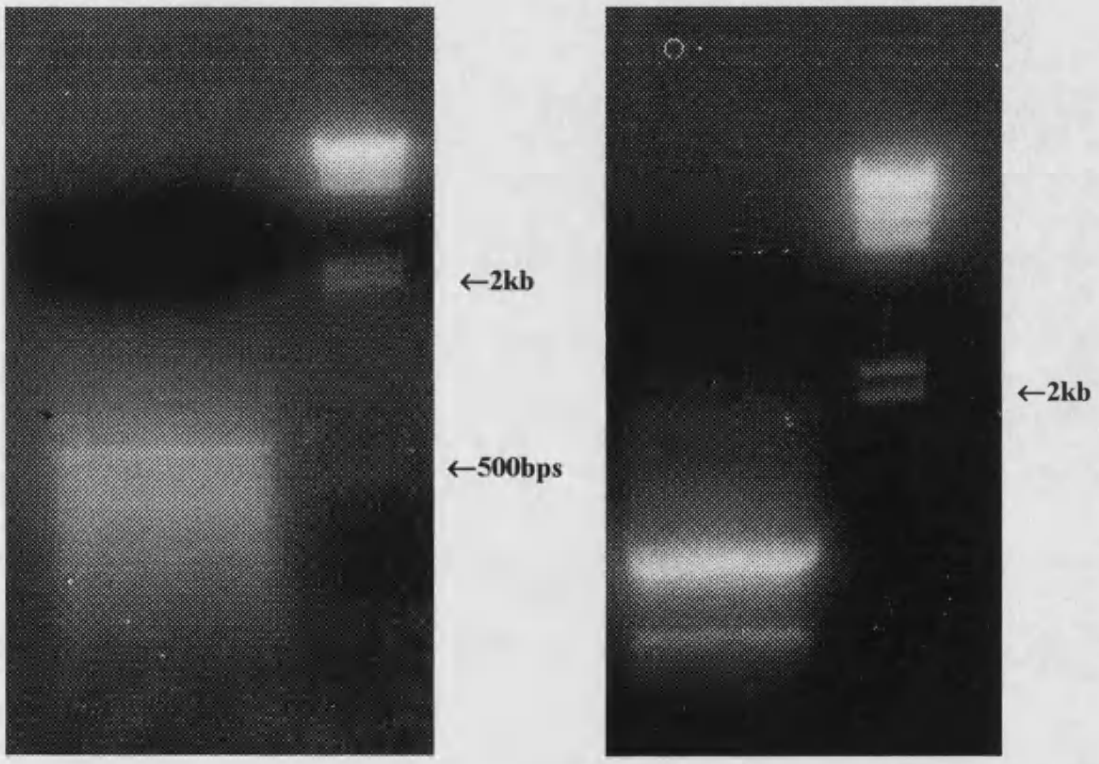
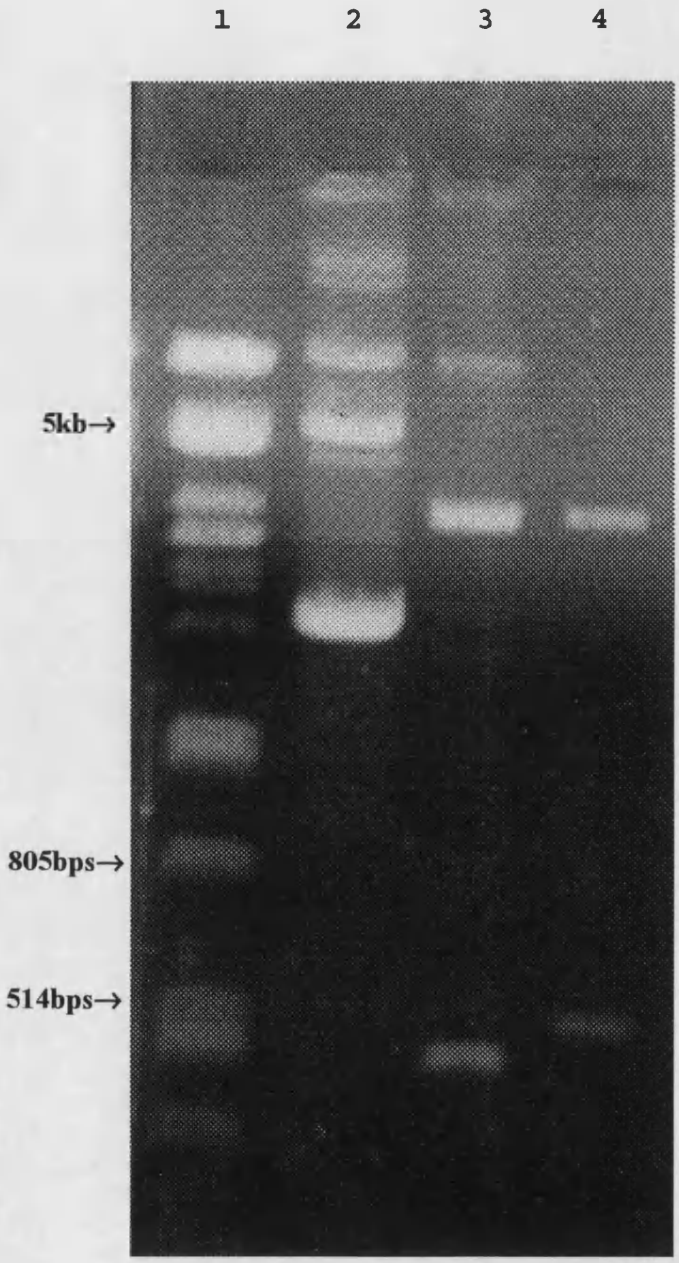


Figure 39. Restriction analysis of transformants.

Miniprep DNA was restricted with BamHI and XbaI and electrophoresed through a 1% agarose gel. Lane (1) λPstI size marker, (2) uncut pBluescript™, (3) clone 1, (4) clone 2.



6.2.1.3 Sequence analysis

Clone 2 was a truncated form of clone 1, missing sequence from both 3' and 5' ends. This variation in size probably results from differential digestion by DNA polymerase 1 during insert preparation before ligation. The nucleic acid sequence and predicted translated product of the larger cDNA are shown in Figure 40. This amino acid sequence was used to search the EMBL and wormpep databases with the GCG TFASTA programme (Devereux *et al.* 1984) . The closest matches reported are presented as a table of identities (Table 4) and a sequence alignment. The derived amino acid sequence shares a high level of identity with a *C. elegans* partial cDNA and the rat L9 Ribosomal protein (86% over 119 amino acids and 61% over 138 amino acids respectively), suggesting that this clone (now termed HCL9) and all the other sequences in the alignment are orthologues of the L9 ribosomal protein. The antisense PCR primer has annealed at a site within the coding sequence of HCL9 producing a truncated product lacking 3' sequence and the stop codon. The SL1 splice-leader is present at the 5' end of the cDNA, but the A2 primer is found at the 3' end. A1 would be expected at this position as it was used as an antisense primer in the second round of the PCR. A likely explanation of this anomaly is that an excess of first round reaction products were present in the second round of the nested PCR. The template would then have been amplified in a mainly unidirectional and non-exponential manner from the SL1 primer. Additionally, the A2 primer carried over with the template could have caused priming in the reverse direction, accounting for the 3' A2 sequence.

Figure 40 Nucleotide and predicted amino acid sequence of the HCL9 cDNA.

The SL1 splice-leader and A2 primer sequences are marked in italics and the predicted start codon is in bold.

```
    GTTTAATTACCCAAGTTTGAGGTAATCCGGGAAGATCATGAAGCTGGTCGAGTCCAATGA
  1 ---------+---------+---------+---------+---------+---------+
    CAAATTAATGGGTTCAAACTCCATTAGGCCCTTCTAGTACTTCGACCAGCTCAGGTTACT

                                          M  K  L  V  E  S  N  D

    TACCGTAGATTTTCCTGATGGTGTGACGTTCACCGTCAAAAACCGCGTAGTCCACGTTAC
 61 ---------+---------+---------+---------+---------+---------+
    ATGGCATCTAAAAGGACTACCACACTGCAAGTGGCAGTTTTTGGCGCATCAGGTGCAATG

     T  V  D  F  P  D  G  V  T  F  T  V  K  N  R  V  V  H  V  T

    CGGACCTCGCGGAACACTCAAACGTGACTTCCGCCACCTTCACATGGAGATGGAACGTGT
121 ---------+---------+---------+---------+---------+---------+
    GCCTGGAGCGCCTTGTGAGTTTGCACTGAAGGCGGTGGAAGTGTACCTCTACCTTGCACA

     G  P  R  G  T  L  K  R  D  F  R  H  L  H  M  E  M  E  R  V

    CGGGAAGAACCAGCTGCGTGTACGCAAGTGGTTTGGAGTTCGCAAAGAGATCGCTGCCAT
181 ---------+---------+---------+---------+---------+---------+
    GCCCTTCTTGGTCGACGCACATGCGTTCACCAAACCTCAAGCGTTTCTCTAGCGACGGTA

     G  K  N  Q  L  R  V  R  K  W  F  G  V  R  K  E  I  A  A  I

    TCGAACAGTGTGCTCACACATCCAGAACATGATCAAGGGTGTCACCAAGGGTTTCCGCTA
241 ---------+---------+---------+---------+---------+---------+
    AGCTTGTCACACGAGTGTGTAGGTCTTGTACTAGTTCCCACAGTGGTTCCCAAAGGCGAT

     R  T  V  C  S  H  I  Q  N  M  I  K  G  V  T  K  G  F  R  Y

    CAAGATGCGATCCGTATACGCCCATTTCCCCATCAACGTCACTCTTCAAGATGGTGGAAG
301 ---------+---------+---------+---------+---------+---------+
    GTTCTACGCTAGGCATATGCGGGTAAAGGGGTAGTTGCAGTGAGAAGTTCTACCACCTTC

     K  M  R  S  V  Y  A  H  F  P  I  N  V  T  L  Q  D  G  G  R

    AACTGTTGAGATTCGTAACTTCCTTGGAGAAAAGATTGTTCGTCCTGTGCCCCTCCGGAT
361 ---------+---------+---------+---------+---------+---------+
    TTGACAACTCTAAGCATTGAAGGAACCTCTTTTCTAACAAGCAGGACACGGGGAGGCCTA

     T  V  E  I  R  N  F  L  G  E  K  I  V  R  P  V  P  L  R  M

    GTGTCACTGCCACGTTGTCCACTTCACAAGGACGCTCGAT
421 ---------+---------+---------+---------+ 460
    CACAGTGACGGTGCAACAGGTGAAGTGTTCCTGCGAGCTA

     C  H  C  H  V  V  H  F  T  R  T  L  D  -
```

Table 4. Results of TFASTA search with HCL9, table of identities.

The EMBL and Wormpep databases were queried with the predicted amino-acid sequence of HCL9, using the GCG "TFASTA" program. The four closest matches reported are displayed. Percentage identities were calaculated using the GCG computer programme DISTANCES (Devereux *et al.* 1984).

Species	Accession number	U09953	D76246	X51706	X91958
H. contortus	HCL9	**58%**	**86%**	**59%**	**48%**
Human Expressed Sequence Tag	U09953		**63%**	**98%**	**56%**
C. elegans EST	D76246	**63%**		**63%**	**47%**
Rat EST	X51706	**98%**	**63%**		**57%**
A. thaliana EST	X91958	**56%**	**47%**	**57%**	

Figure 41. Sequence alignment of the predicted amino-acid sequence of HCL9 with other L9 ribosomal proteins.

The amino acid sequences above (Table 4) were aligned with HCL9 using the GCG multiple alignment programme PILEUP (Devereux *et al.* 1984).

```
                                                        40
  HCL9  MKLVESNDTV DFPDGVTFTV KNRVVHVTGP RGTLKRDFRH
x51706  MKTILSNQTV DIPENVDITL KGRTVIVKGP RGTLRRDFNH
u09953  MKTILSNQTV EIPENVDITL KGRTVIVKGP RGTLRRDFNH
d76246  MKLIESNDTV VFPEGVTFTV KNRIVHVTGP RGTIRKDFRH
x91958  MKTILSSETM DIPDSVTIKV HAKVIEVEGP RGKLVRDFKH

        41                                          90
  HCL9  LHMEMERV.. .GKNQ..LRV RKWFGVRKEI AAIRTVCSHI QNMIKGVTKG
x51706  INVELSLL.. .GKKKKRLRV DKWWGNRKEL ATVRTICSHV QNMIKGVTLG
u09953  INVELSLL.. .GKKKKRLRV DKWWGNRKEL ATVRTICSHV QNMIKGVTLG
d76246  LHMEMERI.. .GKST..LRV RKWFGVRKEL AAIRTVCSHI KNMIKGVTVG
x91958  LNLDFQLIKD PETGKKKLKI DSWFGTRKTS VSIRTALRHV DNLISGVTRG

        91                                         140
  HCL9  FRYKMRSVYA HFPINVTLQD GGRTVEIRNF LGEKIVRPVP LRM.CHCHVV
x51706  FRYKMRSVYA HFPINVVIQE NGSLVEIRNF LGEKYIRRVR MRT.GVACSV
u09953  FRYKMRSVYA HFPINVVIQE NGSLVEIRNF LGEKYIRRVR MRP.GVACSV
d76246  FRYKMRSVYA HFPINVTLQD GNRTVEIR.. .......... ..........
x91958  FRYKMRFVYA HFPINVSIGG DGSLIEIRNF LGEKKVRKVE MLDWCNHCSD

        141
  HCL9  HFTRTLD
x51706  SQAQKDEL
u09953  SQAQKDEL
d76246  ........
x91958  PEKVKDEI
```

6.2.2 Cloning of the 5' end of Hg2/3.

To improve the specificity of the reaction and prevent the amplification of spurious products, touchdown PCR conditions were used. Much less template was used in subsequent second round reactions (0.5µl of first round product diluted 1:10) to prevent any further problems with primer carry-over. To avoid time consuming and expensive cloning and sequencing of candidate PCR products, an aliquot of final PCR products was analysed by southern blotting with an oligonucleotide (A0) designed to an upstream region of the known Hg2/3 sequence (Figure 36 and Table 3). Total RNA was extracted from both egg and adult tissue (Figure 42). Poly $A^{(+)}$ RNA was purified from 200µgs of each RNA sample and used as a template for cDNA transcription. A sample (1ul) of cDNA was used as a template in a control PCR reaction with the degenerate primers DL11 and DL19 which were initially used to clone the first partial Hg2/3 clone (Laughton 1993), reaction products were electrophoresed through a 1.2% agarose gel (Figure 43). The heterologous product of around 450bps suggested that the cDNA sample may have contained transcripts encoding inhibitory amino acid receptor subunits.

A diffuse band of approximately 500bps was produced in the first round of the hemi-nested Hg2/3 amplification from egg cDNA (Figure 44). The second round reaction yielded a single product of approximately 500bps. Southern blotting of PCR products with end-labelled A0 showed a band of a similar size (Figure 45). This was subcloned as before and three clones (Hg2F1-3) were sequenced in both directions. The three nucleic acid sequences code for the same amino acid sequence. The confirmed sequence of Hg2F3 is shown in Figure 46, whereas Table 5 shows the nucleotide differences between Hg2F3 and the other two clones.

Figure 42. Agarose gel electrophoresis of total RNA extracted from egg and adult *H. contortus*.

Samples (3µl) of RNA extracted from both egg (lane 1.) and adult (lane 2.) tissue were electrophoresed through a 1% agarose gel.

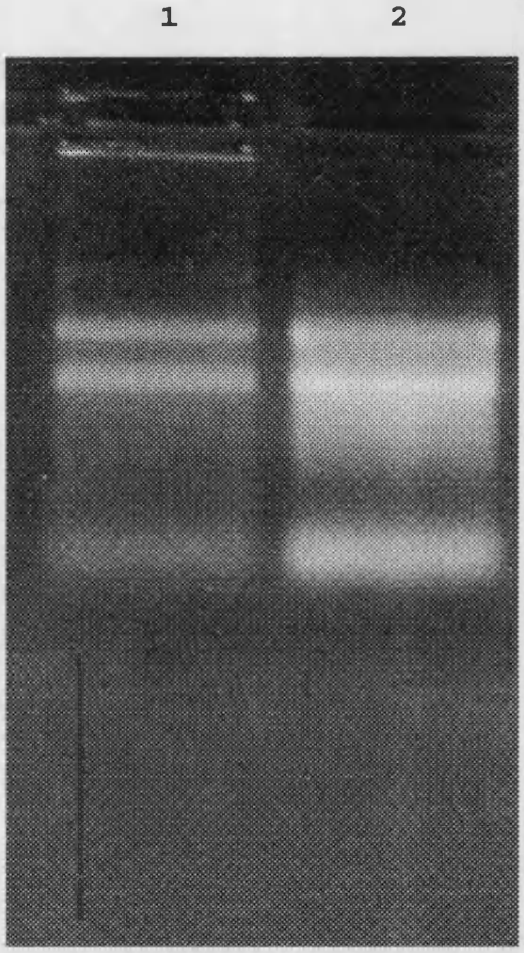


Figure 43. Control amplification using new cDNA template.

cDNA was amplified using the primers DL11 and DL19 which were used to amplify the original 450bp partial clones Hg1-5 (see chapter 1. and Laughton 1993). After a standard heat denaturation, the samples were cycled through 40 cycles of denaturing (95°C/45secs), annealling (45°C/90secs), extension (72°C/90secs). Electrophoresis through a 1.2% agarose gel revealed a diffuse product of the expected size (450bps) produced from egg cDNA but not from adult. Lane (1) λPstI, (2) 10μl of PCR reaction from egg cDNA template.

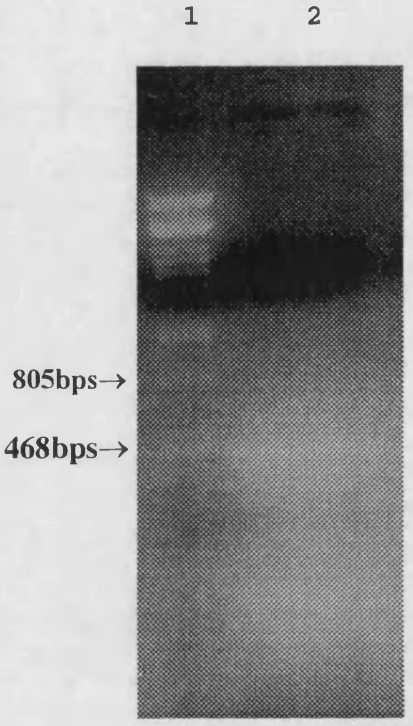


Figure 44. Agarose gel electrophoresis of first round products from the nested touchdown PCR.

Egg and adult cDNA (2µl) was used as a template in each of the first round hemi-nested PCR reactions with the primers SL1 and A2. The samples were cycled through 50 cycles of touchdown thermocycling conditions. Annealling (60°C/ 60secs - falling by 1°C every two cycles to a final temperature of 50°C), extension (72°C/90secs), denaturing (95°C/30secs). *Taq* DNA polymerase was used (3.75 units), 10µl of each of the reaction mixtures was electrophoresed through a 1.2% agarose gel for analysis. Lane (1) λPstI marker, (2&3) adult cDNA template, 2 and 4mM $MgCl_2$ respectively, (4-6) egg cDNA 2,4 and 6mM $MgCl_2$.

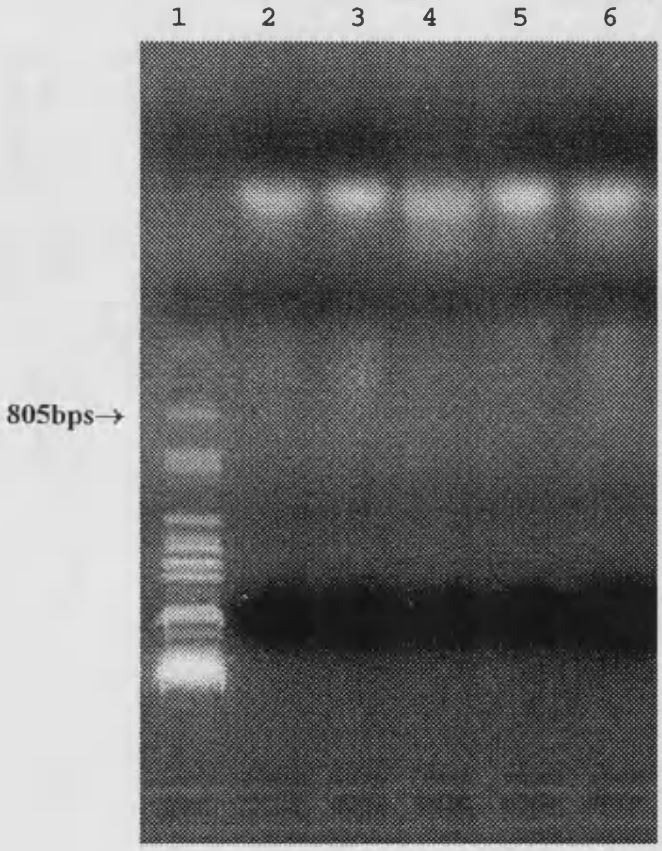
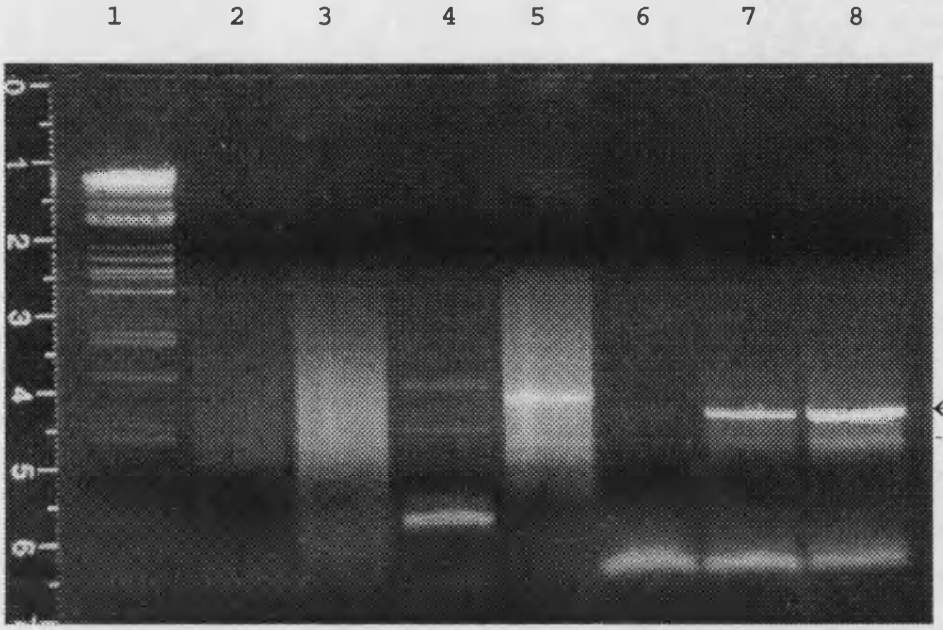


Figure 45. Agarose gel electrophoresis of second round products of nested PCR and corresponding southern blot.

PCR conditions were as described in Figure 44. Lane (1) λPstI marker, (2) positive control for Southern blot - A0 polymerised using low temperature PCR, (3) negative control for Southern blot - 5μg herring sperm DNA, (4) SL1 single primer control PCR, (5) A1 single primer control PCR, (6-8) PCR using egg cDNA template - 6,4 and 2mM $MgCl_2$.



Southern blot of previous gel, lane order as above.

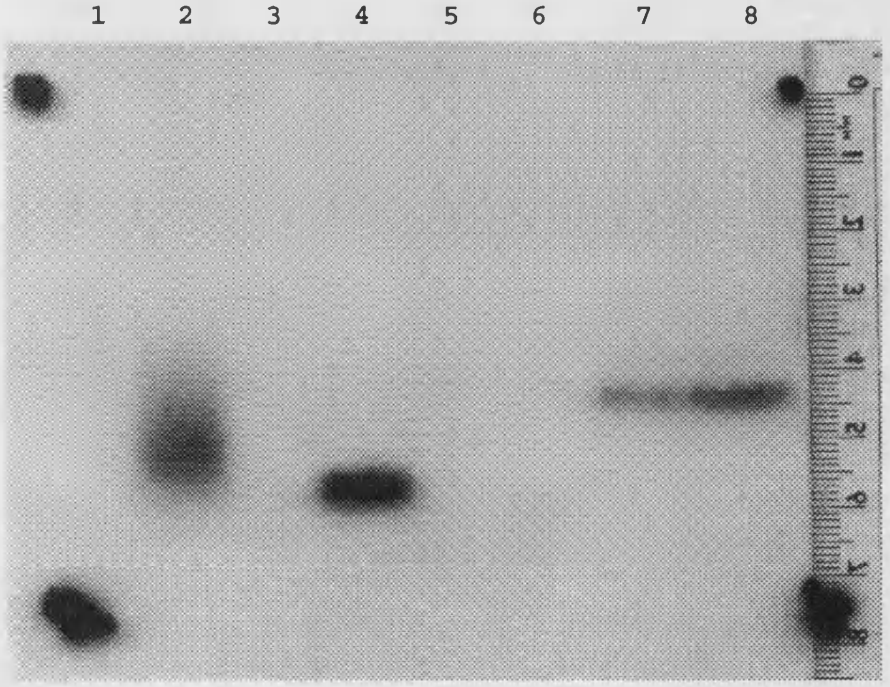


Table 5 Sequence differences between Hg2F3 and Hg2F1&2.

Nucleotide and amino acid changes shown are numbered according to the Hg2F3 sequence, substitutions shown are those found in Hg2F1 or 2 but not present in Hg2F3.

Nucleotide residue	Nucleotide substitution	Amino acid substitution
37	A to G(Hg2F1 and Hg2F2)	NONE
88	A to G(Hg2F1 only)	NONE

The 3' end of the Hg2F3 nucleic acid sequence is very similar to the 5' end of the partial Hg2/3 sequence (Hg2trun) cloned by Laughton (1993). Only three nucleotide sequences are seen in Hg2F3 which would result in one conservative amino-acid change (109Tyr-Phe - Figure 46) . This demonstrates that these two partial clones are fragments of the same full-length sequence, shown in Figure 47. The nucleic and amino acid differences probably arise through variation between the *H. contortus* populations from which the template cDNAs were made. Polymorphic variation between and within populations of *H. contortus* is well documented. Kwa *et al.* (1993) found considerable restriction fragment polymorphism between populations of benzimidazole sensitive and resistant populations of *H. contortus.* In addition, many single nucleotide differences between individual cDNA clones derived from the same population of *H. contortus* have been observed in this laboratory (Laughton *et al.* 1994, N. Delaney Personal Communication)

The predicted full-length Hg2/3 cDNA (Figure 47) shares a high identity with and similar organisation to Cegbr2/3, although Hg2/3 has a long (70bp) 5' untranslated region whereas the predicted start codon of Cegbr2/3 is immediately vicinal to the SL1 splice-leader sequence. There is a possible N-glycosylation site (Figure 47, marked with an arrow) which is conserved in both Cegbr2/3 and Gluclβ.

Figure 46. Sequence of Hg2F3 partial cDNA clone.

The SL1 and A1 primer sequences are in italics and the predicted start codon is in bold.

```
   GGTTTAATTACCCAAGTTTGAGTTTCTGACTTTTACACTTGGAATAGCAGATGCTTCATC
 1 ---------+---------+---------+---------+---------+---------+
   CCAAATTAATGGGTTCAAACTCAAAGACTGAAAATGTGAACCTTATCGTCTACGAAGTAG

   TAATGGCATCGATCTAAAGGCTGCAGGACAAGGCATGCGCAATTCCGTCCCTCTGGCGAC
61 ---------+---------+---------+---------+---------+---------+
   ATTACCGTAGCTAGATTTCCGACGTCCTGTTCCGTACGCGTTAAGGCAGGGAGACCGCTG

                                     M  R  N  S  V  P  L  A  T

    TCGAATAGGGCCTATGCTGGCCCTTATCTGTACCGTCAGTACAATTATGTCCGCAGTAGA
121 ---------+---------+---------+---------+---------+---------+
    AGCTTATCCCGGATACGACCGGGAATAGACATGGCAGTCATGTTAATACAGGCGTCATCT

     R  I  G  P  M  L  A  L  I  C  T  V  S  T  I  M  S  A  V  E

    GGCCAAGAGGAAACTCAAAGAACAGGAGATTATCCAACGTATTCTCAATAATTACGATTG
181 ---------+---------+---------+---------+---------+---------+
    CCGGTTCTCCTTTGAGTTTCTTGTCCTCTAATAGGTTGCATAAGAGTTATTAATGCTAAC

     A  K  R  K  L  K  E  Q  E  I  I  Q  R  I  L  N  N  Y  D  W

    GAGAGTCAGGCCGAGGGGATTAAATGCTTCCTGGCCAGATACTGGTGGTCCTGTGCTGGT
241 ---------+---------+---------+---------+---------+---------+
    CTCTCAGTCCGGCTCCCCTAATTTACGAAGGACCGGTCTATGACCACCAGGACACGACCA

     R  V  R  P  R  G  L  N  A  S  W  P  D  T  G  G  P  V  L  V

    GACGGTAAACATCTATTTGCGTTCAATTTCAAAAATCGATGACGTTAATATGGAGTACAG
301 ---------+---------+---------+---------+---------+---------+
    CTGCCATTTGTAGATAAACGCAAGTTAAAGTTTTTAGCTACTGCAATTATACCTCATGTC

     T  V  N  I  Y  L  R  S  I  S  K  I  D  D  V  N  M  E  Y  S

    TGCTCAATTTACTTTTCGAGAAGAATGGGTGGATGCTAGGCTTGCCTACGGCCGTTTCGA
361 ---------+---------+---------+---------+---------+---------+
    ACGAGTTAAATGAAAAGCTCTTCTTACCCACCTACGATCCGAACGGATGCCGGCAAAGCT

     A  Q  F  T  F  R  E  E  W  V  D  A  R  L  A  Y  G  R  F  E

    GGACGAATCCACGGAGGTGCCGCCGTTCGTAGTGTTGGCGACCAGCGAGAATGCCGACCA
421 ---------+---------+---------+---------+---------+---------+
    CCTGCTTAGGTGCCTCCACGGCGGCAAGCATCACAACCGCTGGTCGCTCTTACGGCTGGT

     D  E  S  T  E  V  P  P  F  V  V  L  A  T  S  E  N  A  D  Q

    GTCACAACAGATTTGGATGCCGGACACATTCTTCCAAAATGAAAAAGTGGCACGACGACA
481 ---------+---------+---------+---------+---------+---------+
    CAGTGTTGTCTAAACCTACGGCCTGTGTAAGAAGGTTTTACTTTTTCACCGTGCTGCTGT

     S  Q  Q  I  W  M  P  D  T  F  F  Q  N  E  K  V  A  R  R  H
```

```
    TCTCATAGACAAGCC
541 ---------+----- 555
    AGAGTATCTGTTCGG

     L  I  D  K
```

Figure 46 Alignment of Hg2F3 with original partial sequence (Hg2trun).

Alignment of the overlapping region of the two Hg2/3 clones. Polymorphisms are highlighted in bold.

```
          1                                                  50
Hg2Trun   CCAGACACCTAC TTCCAAAA TGAAAAAGTG GCACGACGAC ATCTCATAGA
  Hg2F3   CCGGACACATTC TTCCAAAA TGAAAAAGTG GCACGACGAC ATCTCATAGA
          P  D  T  Y/F F  Q  N   E  K  V   A  R  R  H   L  I  D

          51
Hg2trun   CAAGCC
  Hg2F3   CAAGCC
           K  P
```

Figure 47. Full-length predicted amino-acid sequence of Hg2/3

The predicted intron is in italics, putative transmembrane domains are underlined. The probable N-glycosylation site is marked with an arrow.

```
                                        ↓
  1  KRKLKEQEII QRILNNYDWR VRPRGLNASW PDTGGPVLVT VNIYLRSISK

 51  IDDVNMEYSA QFTFREEWVD ARLAYGRFED ESTEVPPFVV LATSENADQS

101  QQIWMPDTFF QNEKEARRHL IDKPNVLIRI HKDGSILYSV RLSLVLSCPM

151  SLEFYPLDRQ NCLIDLASYG YTTQDIKYEW KEQNPVQQKD GLRQSLPSFE

201  LQDVVTKYCT SKTNTGEYSC LRTQMVLRRE FSYYLLQLYI PSFMLVIVSW
                                                  TM1
251  VSFWLDKDSV PARVTLGVTT LLTMTTQSSG INANVPPVSY TKAIDVWIGV
                          TM2
301  CLAFIFGALL EFAWVNYAAR KDMSCGQRMM KQLPQDGYST ASRQSTTNLV
           TM3
351  LLSHIRSTPQ RAFETYRRRL PPGVPYWICL FQCVILGCLL DVKHGACSTR

401  SSVRPSVLIV PPNSPRLTFL LSILRLVAFH LTQCLISRL* GEYSCARVKL
                               TM4
451  LLRREYSYYL IQLYIPCIML LVVSWVSFWL DKDAVPARVS LGVTTLLTMT
                    TM1                       TM2
501  TQASGINSKL PPVSYIKVQA VDVWIGVCLA FIFGALLEYA VVNYYGRKEF
                                    TM3
551  LRKEKKKKTR LDDCVCPSER PALRLDLSNY RRRGWRPLNR LLDMLGRNAY

601  LSRRVDLMSR ITFPSLFTAF LVFYYSVYVK QSNLD*
                    TM4
```

Table 6. Amino acid identity of Hg2 and Hg3 with related TGIC subunits.

Subunit	Description	Hg2	Hg3	Cegbr2	GluClα	GluClβ	U28803
Hg2	*H. contortus* putative GluCl subunit	*	77%	79%	53%	46%	33%
Hg3	*H. contortus* putative GluCl subunit	77%	*	77%	56%	48%	37%
U41113	*C. elegans* Cegbr3 putative GluCl subunit	72%	87%	82%	57%	45%	35%
U40573	*C. elegans* Cegbr2 putative GluCl subunit	79%	77%	*	55%	47%	37%
U14524	*C. elegans* GluClα subunit	53%	56%	55%	*	44%	32%
U14525	*C. elegans* GluClβ subunit	46%	48%	47%	44%	*	33%
U28803	*Aedes aegypti* (yellow fever mosquito) $GABA_A$ receptor subunit.	33%	37%	37%	32%	33%	*
X52800	Human glycine receptor α_2 subunit	40%	44%	42%	38%	37%	40%

Figure 48. Sequence alignment of Hg2 and Hg3 with other inhibitory TGIC subunits.

The amino acid sequences above (*Table 6*) were aligned with Hg2/3 using the GCG multiple alignment programme PILEUP (Devereux *et. al.* 1984). Conserved cysteine residues are marked with an asterisk, potential serine and threonine phoshorylation sites are denoted in bold, the approximate positions of the predicted transmembrane domains are underlined

```
          1                                                  50
   Hg2    .......... .......... .......MRN SVPLATRIGP MLALICTVST
   Hg3    .......... .......... .......MRN SVPLATRIGP MLALICTVST
Cegbr3    .......... .......... .......... ......MWHY RLTTILLIIS
Cegbr2    .......... .......... .......... ......MWHY RLTTILLIIS
GluClα    MATWIVGKLI IASLILGIQA QQARTKSQDI FEDDNDNGTT TLESLARLTS
GluClβ    .......... .......... .......... .....MTTPS SFSILLLLLL
u28803    ........MS LEIEVPHVRC PSLGVLILTL NLALFLPQTI NRTPPYVLAG
x52008    .......... MNRQL..... ....VNILTA LFAFFLETNH FRTAFCKDHD

          51                                                 100
   Hg2    IMSAVEAKRK LKEQEIIQRI L...NNYDWR VRPRGLNASW PDTGGPVLVT
   Hg3    IMSAVEAKRK LKEQEIIQRI L...NNYDWR VRPRGLNASW PDTGGPVLVT
Cegbr3    IIHSIRAKRK LKEQEIIQRI L...KDYDWR VRPRGMNATW PDTGGPVLVT
Cegbr2    IIHSIRAKRK LKEQEIIQRI L...KDYDWR VRPRGMNATW PDTGGPVLVT
GluClα    PIHIPIEQPQ TSDSKILAHL F..TSGYDFR VRPPT..... .DNGGPVVVS
GluClβ    MPVVTNGEYS MQSEQEILNA L..LKNYDMR VRPPPANSS. ..TEGAVNVR
u28803    TGGGSMLGD. VNISAILDSF ...SVGYDKR VRPNY..... ..GGPPVEVG
x52008    SRSGKQPSQT LSPSDFLDKL MGRTSGYDAR IRPNF..... ..KGPPVNVT

          101                                                150
   Hg2    VNIYLRSISK IDDVNMEYSA QFTFREEWVD ARLAYGRFED ES.TEVPPFV
   Hg3    VNIYLRSISK IDDVNMEYSA QFTFREEWVD ARLAYGRFED ES.TEVPPFV
Cegbr3    VNIYLRSISK IDDVNMEYSA QFTFREEWTD QRLAYERYEE SGDTEVPPFV
Cegbr2    VNIYLRSISK IDDVNMEYSA QFTFREEWTD QRLAYERYEE SGDTEVPPFV
GluClα    VNMLLRTISK IDVVNMEYSA QLTLRESWID KRLSYGVKGD GQ....PDFV
GluClβ    VNIMIRMLSK IDVVNMEYSI QLTFREQWID PRLAY...EN LGFYNPPAFL
u28803    VTMYVLSISS VSEVLMDFTL DFYFRQFWTD PRLAYRKRPG VETLSVGSEF
x52008    CNIFINSFGS VTETTMDYRV NIFLRQQWND SRLAYSEYPD .DSLDLDPSM

          151                                                200
   Hg2    VLATSENADQ SQQIWMPDTF FQNEKEARRH LIDKPNVLIR IHKDGSILYS
   Hg3    VLATSENADQ SQQIWMPDTF FQNEKEARRH LIDKPNVLIR IHKDGSILYS
Cegbr3    VLATSENADQ SQQIWMPDTF FQNEKEARRH LIDKPNVLIR IHKNGQILYS
Cegbr2    VLATSENADQ SQQIWMPDTF FQNEKEARRH LIDKPNVLIR IHKNGQILYS
GluClα    ILTV...... GHQIWMPDTF FPNEKQAYKH TIDKPNVLIR IHNDGTVLYS
GluClβ    TVPHVKKS.. ...LWIPDTF FPTEKAAHRH LIDMENMFLR IYPDGKILYS
u28803    I......... .KNIWVPDTF FVNEKQSYFH IATTSNEFIR VHHSGSITRS
x52008    L......... .DSIWKPDLF FANEKGANFH DVTTDNKLLR ISKNGKVLYS
```

```
          201                                                  250
   Hg2  VRLSLVLSCP MSLEFYPLDR QNCLIDLASY GYTTQDIKYE WKEQ.NPVQQ
   Hg3  VRLSLVLSCP MSLEFYPLDR QNCLIDLASY GYTTQDIKYE WKEQ.NPVQQ
Cegbr3  VRLSLVLSCP MSLEFYPLDR QNCLIDLASY AYTTQDIKYE WKEK.KPIQQ
Cegbr2  VRLSLVLSCP MSLEFYPLDR QNCLIDLASY AYTTQDIKYE WKEK.KPIQQ
GluClα  VRISLVLSCP MYLQYYPMDV QQCSIDLASY AYTTKDIEYL WKEH.SPLQL
GluClβ  SRISLTSSCP MRLQLYPLDY QSCNFDLVSY AHTMNDIMYE WDPS.TPVQL
u28803  IRLTITASCP MGLQYFPMDR QLCHIEIESF GYTMRDIRYF WKDGLSSVGM
x52008  IRLTLTLSCP MDLKNFPMDV QTCTMQLESF GYTMNDLIFE WLSD.GPVQV
                 *                 *

          251                                                  300
   Hg2  KDGLRQSLPS FELQDVVTKY .CTSKTNTGE YSCLRTQMVL RREFSYYLLQ
   Hg3  KDGLRQSLPS FELQDVVTKY .CTSKTNTGE YSCARVKLLL RREYSYYLIQ
Cegbr3  KDGLRQSLPS FELQDVVTDY .CTSLTNTGE YSCARVVLRL RREYSYYLIQ
Cegbr2  KDGLRQSLPS FELQDVVTDY .CTSLTNTGE YSCLRTRMVL RREFSYYLLQ
GluClα  KVGLSSSLPS FQLTNTSTTY .CTSVTNTGI YSCLRTTIQL KREFSFYLLQ
GluClβ  KPGVGSDLPN FILKNYTTNA DCTSHTNTGS YGCLRMQLLF KRQFSYYLVQ
u28803  SSEV..ELPQ FRVLGHRQRA .TEINLTTGN YSRLACEIQF VRSMGYYLIQ
x52008  AEGL..TLPQ FILKEEKELG YCTKHYNTGK FTCIEVKFHL ERQMGYYLIQ
                              *          *

          301                                                  350
   Hg2  LYIPSFMLVI VSWVSFWLDK DSVPARVTLG VTTLLTMTTQ SSGINANVPP
   Hg3  LYIPCIMLLV VSWVSFWLDK DAVPARVSLG VTTLLTMTTQ ASGINSKLPP
Cegbr3  LYIPCIMLVV VSWVSFWLDK DAVPARVSLG VTTLLTMTTQ ASGINTKLPP
Cegbr2  LYIPSFMLVI VSWVSFWLDK DSVPARVTLG VTTLLTMTTQ SSGINANVPP
GluClα  LYIPSCMLVI VSWVSFWFDR TAIPARVTLG VTTLLTMTAQ SAGINSQLPP
GluClβ  LYAPTTMIVI VSWVSFWIDL HSTAGRVALG VTTLLTMTTM QSAINAKLPP
u28803  IYIPSGLIVI ISWVSFWLNR DATPARVALG VTTVLTMTTL MSSTNAALPK
x52008  MYIPSLLIVI LSWVSFWINM DAAPARVALG ITTVLTMTTQ SSGSRASLPK
                TM1                          TM2

          351                                                  400
   Hg2  VSYTK..AID VWIGVCLAFI FGALLEFAWV NYAAR..... .KDMSCGQRM
   Hg3  VSYIKVQAVD VWIGVCLAFI FGALLEYAVV NYYGR..... .KEFLRKEKK
Cegbr3  VSYIK..AVD VWIGVCLAFI FGALLEYAVV NYYGR..... .KEFLRKEKK
Cegbr2  VSYTK..AID VWIGVCLAFI FGALLEFALV NYAAR..... .KDMTQ....
GluClα  VSYIK..AID VWIGACMTFI FCALLEFALV NHIAN..... .KQGV..ERK
GluClβ  VSYVKV..VD VWLGACQTFV FGALLEYAFV SYQDS..... .VRQNDRSRE
u28803  ISYVK..SID VYLGTCFVMV FASLLEYATV GYMAKRIQIG KQRFMAIQKI
x52008  VSYVK..AID IWMAVCLLFV FAALLEYAAV NFVSRQ.... HKEFLRLRR.
                    TM3

          401                                                  450
   Hg2  MKQLPQDGYS TASRQSTTNL VLLSHIRSTP QRAFETYRRR LPPGVPYWIC
   Hg3  KKTRLDDCVC PSERPAL... ....RLDLSN YR....RRGW RP.LNRLLDM
Cegbr3  KKTRIDDCVC PSDRPPL... ....RLDLSA YR....SVKR LPIIKRISEI
Cegbr2  ........VS QRIRQMK... ....QLPTEG YRPLSASQGR SSFCCRI..F
GluClα  ARTEREKAEI PLLQNLHNDV PTKVFNQEEK VRTVPLNRRQ MNSFLNLLET
GluClβ  KAARK....A QRRREKLEMV DAEVYQPPCT CHTFEA...R ETFRDKVRRY
u28803  AEQKKQQAAD ANHPPPPPPV SDHSHGHGHG HSHGHQHTPK QQMGSRSGPL
x52008  .RQKRQNKEE DVTRESRFNF SGYGMGHC.. .......... ..........
```

```
          451                                                       500
   Hg2    L..... ..... ..... ..... .....FQCVI
   Hg3    L..... ..... ..... ..... .....GRNAY
Cegbr3    L..... ..... ..... ..... .....STNID
Cegbr2    V..... ..... ..... ..... .....RRYKE
GluClα    K..... ..... ..... ..... .....TEWND
GluClβ    F..... ..... ..... ..... .....TKPDY
u28803    FQEVRFKVHD PKAHSKGGTL ENTINGGRGG GGPPGGGGGP PGGGGGGPDE
x52008    .....LQVKD GTA..... .....VK ATPANPLPQP PKDGDA....

          501                                                       550
   Hg2    LGCLLDVKHG ACSTRSSVRP SVLIVPPNSP RLTFLLSILR LVAFHLTQCL
   Hg3    LSRRVDLM.. ..... .....S RITFPSLFTA FLVFYYSVYV
Cegbr3    ISRRVDLM.. ..... .....S RLTFPLTFFS FLIFYYVAYV
Cegbr2    RSKRIDVV.. ..... .....S RLVFPIGYAC FNVLYWAVYL
GluClα    ISKRVDLI.. ..... .....S RALFPVLFFV FNILYWSRFG
GluClβ    LPAKIDF... ..... .....YA RFVVPLAFLA FNVIYWVSCL
u28803    ESGAPQHLIH PGKDINKLLG ITPSDIDKYS RIVFPVCFVC FNLMYWIIYL
x52008    ..... ....IKKKFV DRAKRIDTIS RAAFPLAFLI FNIFYWITYK
                                      TM4

          551          568
   Hg2    ISRL..... .....
   Hg3    KQSNLD*... .....
Cegbr3    KQSRD*.... .....
Cegbr2    M*..... .....
GluClα    QQNVLF*... .....
GluClβ    IMSANASTPE SLV*....
u28803    HVSDVVADDL VLLGEEK*
x52008    IIRHEDVHKK *.....
```

6.2.3 Attempted full length amplification of Hg2/3

Primers were designed to the 5' and 3' ends of the predicted coding sequence of Hg2/3. This nucleotide sequence was scanned for restriction sites using the MAP programme from the GCG program suite (Devereux *et al.* 1984). No restriction sites were found for the enzymes NotI and XbaI so their recognition sequences were included at the 5' end of the sense and antisense primers respectively. A degenerate antisense primer was also synthesised to allow for possible DNA polymorphisms between the cDNA pools from which Hg2F3 and the original truncated sequence were cloned.

Table 7. Oligonucleotide primers designed for the amplification of the full length Hg2/3 cDNA.

Oligonucleotide	Sequence 5'-3' (Restriction sites in bold)
CS1	ATCGTT**GCGGCCGC**ATGCGCAATTCCGTCCCTCTGG
CS2	ATCTT**GCGGCCGC**ATGCGCAATTCCGTCCCTCTGGC
CA1	GTCAGC**TCTAGA**GTCAGTCGAGGTTGCTTTGTTTCA
CA2	CCGTAA**TCTAGA**GTCAGTCGAGGTTGCTTTGTTTC
CAD3	CCGTAA**TCTAGA**GTCAGTCGAG(GA)TT(GA)CT(TC)TG(TC)TT

A range of touchdown and standard PCR protocols were employed, using both *Taq* DNA polymerase and "Xpand™" enzyme mix, with annealling temperatures from 40-70°C. No products of the expected size were seen with either enzyme using any combination of sense and antisense primers.

6.3 Discussion

The Cegbr2/3 gene product is alternatively spliced to give two mRNA species encoding two putative glutamate receptor subunits which share a common extracellular domain but have different channel-forming regions (**Figure 8**). The Hg2/3 gene codes for two similar subunits, depending on whether the region of the primary transcript encoding the C-terminal channel forming domain of Hg2 is spliced out or not.

The failure of the attempted full length amplifications is probably attributable in part to the rarity of the target message. Even with gene-specific primers, the amplification of other full-length invertebrate TGIC subunit cDNAs has proved difficult (Eastham *et al.* 1997). The inclusion of long restriction sites and anchor sequence may adversely affect the annealling of primers to their target sequences. As the 5' and 3' end sequences were derived from different populations it is possible that there are polymorphisms between them at these sites which would exacerbate this problem.

Likely extracellular and transmembrane domains were determined by sequence comparison and hydrophilicity analysis. The two putative subunits differ in their TM2 domains; Hg3 has a serine to alanine substitution. Cegbr2 and Cegbr3 have identical TM2 domains to Hg2 and Hg3 respectively, this substitution is found in GABA receptor subunits cloned from dieldrin resistant strains of both *D. melanogaster* and *Aedes aegyptii* (Ffrench-Constant *et al.* 1991, Thompson *et al.* 1993), however the physiological role of the substitution in nematode species is unclear. The predicted TM4 region of Hg2 is markedly different from that predicted in other known sequences, the hydrophilic histidine within this region (His_{545} -Figure 48) was originally considered to be a PCR induced mutation as other sequences have a conserved tyrosine at this position. These two amino acids are encoded by very similar codons (CAC-His, TAC-Tyr) so a single point mutation could have caused this substitution. Subsequent sequencing of genomic DNA (S.Jagannathan unpublished results) has shown that this histidine is genuine.

Hg3 and Cegbr3 share 87% amino acid identity (Table 7), the only region at which the two amino acid sequences show any major divergence is in the intracellular domain immediately proximal to the predicted TM4 sequence (Figure 48 residues 432-500). The intracellular domain of TGIC receptor subunits is typically the least conserved (Barnard 1992). Hg2 and Cegbr2 share 79% identity and are also very similar at their N-termini, but widely divergent thoughout their intracellular and TM4 regions (residue 394-). A similar relationship exists between Hg2 and Hg3, although Hg2 and Hg3 are more like their *C. elegans* orthologues than each other.

The divergent intracellular loop domains have different phosphorylation sites, the putative intracellular loop of Hg3 and Cegbr3 have possible threonine phosphorylation sites for casein kinase II and cAMP and cGMP dependant protein kinases (Lys-Lys-Lys-Lys-**Thr** 398-403). A protein kinase C site is found immediately C-terminal to this in Hg3 (**Ser**-Glu-Arg 412-414). A second serine residue which may be a substrate for protein kinase C phosphorylation is found close to the predicted TM4 (**Ser**-Arg-Arg 502-504). Hg2 and Cegbr2 lack the casein kinase II and cAMP/cGMP dependant kinase consensus sequence but Hg2 does have four protein kinase C phosphorylation sites in its intracellular domain close to TM4 (**Thr**-Tyr-Arg 436-438, **Ser**-Thr-Arg 513-515, **Ser**-Val-Arg 517-519, **Ser**-Pro-Arg 529-531). These data suggest that Hg2 and Hg3 and Cegbr2 and Cegbr3 may be differently regulated by phosphorylation, however the presence of protein kinase consensus sequences is not sufficient to conclude that these sites are definitely phosphorylated *in vivo*. The possible variation in the ability of these two receptors to act as substrates for protein kinases implies that they may be used interchangeably, perhaps changes in usage are a mechanism in synaptic plasticity. This change may be determined at the mRNA level by the degree of alternative splicing occurring. The physiological role of this unusual splicing mechanism is unclear, several hypotheses can be proposed.

1. *The regulation of splicing occurs as a mechanism of synaptic plasticity.*

2. *The pattern of splicing is under developmental control; the two subunit forms predominate at different stages of the worms life-cycle.*

3. *Different tissues or cells display characteristic splicing patterns resulting in different expression patterns for the two subunits. For example, one neuron may express predominantly Hg2 whereas another may express Hg3 preferentially.*

4. *Both transcripts are produced and the level of splicing is relatively constant. Hg2 and Hg3 are always expressed together and co-assemble as components of a receptor.*

Semi-quantitative PCR showed that Cegbr2 and Cegbr3 transcripts are present in egg, L1-4 and adult stages of *C. elegans* (D. Laughton, Personal Communication.). No gross variations in mRNA abundance were found between the developmental stages suggesting that in *C. elegans* at least, hypothesis 2 does not hold. The two subunits are not expressed at different stages of worm development, although their distribution patterns may change during growth. It can not be

assumed that this model holds for *H. contortus* however and similar experiments should be performed.

The last two hypotheses can be tested easily using immunocytochemical localisation techniques. Antisera should be raised against intracellular epitopes from both subunits and used to study the expression patterns of the two subunits in each stage of the worms lifecycle. If the two antisera are made in different animal species, dual-labelling studies can be performed to confirm regions of co-localisation. Recombinant clones encoding each of the subunits should be constructed and expressed both individually and in tandem in the *Xenopus* system to study the ability of the two subunits to form a receptor. Heteromeric expression with Hg4 and Hg5 subunits from *H. contortus* will determine whether or not Hg2 and Hg3 are capable of assembling as part of an inhibitory glutamate receptor.

7. Final discussion

The putative inhibitory amino acid receptor Hg1 forms a chloride channel gated by high (millimolar) concentrations of glycine when expressed homomerically in Xenopus oocytes (Laughton *et al.* 1994). There is no evidence for glycine acting as a neurotransmitter in nematodes, however homomeric expression of TGIC subunits often results in unphysiological receptor pharmacology (1.3.3.1.2). As Hg1 shares most identity with H27H8.2 and *unc*49, the known GABA receptor subunit, it is more likely to be part of a $GABA_A$ receptor.

An antiserum was raised against a synthetic peptide (HGP1) selected, on the basis of predicted antigenicity and specificity, from the extracellular domain of Hg1. The peptide was conjugated to thryoglobulin to increase its immunogenicity; antibodies to thyroglobulin were subsequently removed by solid phase adsorbtion. The serum titre against the peptide was assayed using the ELISA, giving a half-maximal response at 1:700 dilution. A PCR amplified fragment of Hg1 was cloned into the *E. coli* expression vector pMal, to construct a plasmid which encoded a recombinant fusion protein of the extracellular domain of Hg1 coupled by its N-terminus to the maltose binding protein. SDS-PAGE and western blot analysis of cell lysates derived from transfected *E.coli* showed that the serum also recognised this recombinant form of Hg1, at a dilution of 1:1000.

Immunocytochemical experiments localised HGP1 immunoreactivity to the postsynaptic membrane of the neuromuscular junction in both *A. suum* and *H. contortus*. A number of cephalic neurons also stained in *H. contortus*; a single cell similar to the *C. elegans* sensory neuron AQR and between two and four neurons similar in appearance to the *C. elegans* RI and RM classes. This distribution is consistent with Hg1 forming part of a GABA receptor, assuming the distribution of GABA in the nervous system of *H. contortus* is similar to that described in *C. elegans.*

The 5' end of Hg2/3, a cDNA which appears to encode two putative glutamate receptors by alternative splicing, was cloned using the PCR. The nucleotide sequence of the 3' end of this partial cDNA was nearly identical to that of the 5' end of an existing partial clone (Laughton 1993), the sequences of the two overlapping clones were combined and the nucleotide sequence of the full-length cDNA presented. The derived amino acid sequences of the two predicted protein products share high levels of identity (79% and 87%) with Cegbr2 and Cegbr3, respectively. Hg2 and Hg3 have the additional pair of cysteine residues in their extracellular domain, in common with all members of the GluCl class reported to date (Cully *et al.* 1996). GluClα was

the next closest match identified, sharing 56% identity with Hg3. GluClα_2 has been cloned from *C. elegans* and shares over 80% with GluClα (J. Dent, Personal communication). It is likely therefore, that Hg2 and 3, and the orthologous subunits from *A. suum* and *C. elegans*, are members of a new class of inhibitory glutamate receptor, GluClγ.

The evidence that Hg1 is a GABA receptor subunit is strong, but not conclusive. Nematodes have a large neurotransmitter repertoire, many of these transmitters are myoactive and therefore likely to have receptors on muscle cells. The FaRP PF4 gates a rapidly activating GABA-insensitive chloride channel in denervated *A. suum* muscle (reviewed in Maule *et al.* 1996) and is therefore a candidate ligand for Hg1. Although there is no precedent for peptides acting at receptors of the "cys-loop" TGIC class, there was no precedent for glutamate acting in such a way either. Other FaRPs such as AF1 and AF2 are also inhibitory on denervated *A. suum* muscle strips, although the slower onset of this response suggests it is not mediated by a TGIC. The unequivocal identification of the native ligand for Hg1 will require the co-expression of Hg1 with other subunits known to be expressed at the *H. contortus* NMJ.

The antiserum was raised against a peptide chosen to be unique to Hg1, however the subsequent cloning of Cegbr2/3, which is alternatively spliced to produce two mRNAs encoding related subunits with identical extracellular domains, raises the possibility that Hg1 might have a related subunit sharing the HGP1 sequence. If this were the case the pattern of HGP1 immunoreactivity would not be specific for Hg1, but would reflect the combined expression patterns of these related subunits.

The *C. elegans* cosmid ZC482 has recently been sequenced as part of the genomic sequencing project and the data submitted to the *C. elegans* database at the Sanger centre. TBLASTN analysis of this database, using the N-terminus of Hg1 as a query sequence, showed that ZC482 contains genomic sequence which appears to encode part of a *C. elegans* Hg1 orthologue (Figure 49). To investigate the possibility that this gene undergoes splicing in a manner similar to Cegbr2/3, the database was searched with the predicted TM2 domain of Hg1 (residues 259-281). Two regions which seemed to encode TM2-like domains were located on ZC482 and a neighbouring cosmid BE10, however on further inspection it was found that the TM2-like region on ZC482 was on the opposite strand from the N-terminal sequence, so could not be transcribed as part of the same mRNA. In fact, a well-conserved *C. elegans* Hg1 orthologue is encoded by a region of the genome covering BE10 and ZC482.

Figure 49 Sequence alignment of the N-terminus of Hg1 with cosmid ZC482

The ZC482 sequence contains a 48 nucleotide intron at the point marked with an asterisk. "+" signs in the consensus sequence indicate conservative substitutions. The numbering refers to the nucleotide sequence of the two clones (see Laughton *et al.* 1994 for Hg1 nucleotide sequence).

```
                                                        *
Hg1          90 STYNTTLISYTTKLLNTILLNQDKKFRPTNPDSSPLNVEIDISVRSMGPI
ZC482      6734 TTYNQTLIRYTTKVLDTILLNQDKNFRPVNPDNSPLQVEVDISIRSMGPV
consensus       +TYN TLI YTTK+L+TILLNQDK FRP NPD+SPL VE+DIS+RSMGP+
```

When the peptide HGP1 was designed, very few nematode inhibitory TGIC sequences had been published. Since that time many have been described as part of the *C. elegans* sequencing project or cloned by other methods (1.3.3). Splice variants have been found for many of these subunits (*unc*49, Cegbr2/3, GluClα2). This must be considered when raising antisera against other subunits; the choice of antigen is critical in ensuring the specificity of the antisera. In the case of Hg2 and Hg3, which share a common N-terminal domain, an antigen from the unique C-terminal region must be used. The *C. elegans* sequencing project is extremely useful in this respect, as splice site predictions can be made using the "Genefinder" program, giving predicted protein products from which antigens can be designed.

The work presented in this thesis shows that nematode TGIC subunits can be localised by IC using peptide antisera, however the assumption that such antisera will recognise the native subunit does not always hold. The peptide approach was chosen as it offers excellent specificity, but it is now apparent that the intracellular loops of nematode inhibitory TGIC subunits share very low levels of identity (appendix 1). For this reason, the use of peptide antisera is no longer necessary and good specific antisera could be raised against the whole intracellular loop. Such a strategy would reduce the problem of conformational differences between antigen and native protein, giving more intense and reliable staining. Expression of the recombinant protein as a fusion with the maltose binding protein would avoid the need for conjugation to a carrier protein. Some splice variants of nematode TGICs have been reported with common intracellular loop regions (GluClα_2 - J. Dent Personal communication) so this approach could not be universally applied.

The complete sequence of the Hg2/3 cDNA is presented (Figure 47) and the assumption has been made that it encodes two subunits by alternative splicing. This has now been confirmed; a full length Hg3 cDNA has been cloned and sequenced (S. Jagannathan unpublished results). The relevance of this new class of GluCl subunit remains unclear. Cully *et al.* (1994) showed that

GluClαβ channels expressed in *Xenopus* oocytes had a different EC_{50} (glutamate) and Hill coefficient from those expressed in oocytes injected with *C. elegans* poly $(A)^+$ RNA. This led them to suggest that *in vivo* IGluRs contain at least one further subunit; it is possible that Hg2 and Hg3 perform this function in *H. contortus*.

8. Future work

In order to confirm its native ligand, Hg1 should be co-expressed in *Xenopus* oocytes with *unc*49. *unc*49 is a component of the GABA receptor at the NMJ and Hg1 has an expression pattern consistent with such a function. Ideally the Hg1-like subunit from *C. elegans* should be cloned and used in place of Hg1, however the high level of identity shared by the orthologues should make this unnecessary. The pharmacology of these recombinant channels can then be studied electrophysiologically and compared with the comprehensive pharmacological data published from muscle cells and other nematode tissues.

Full length cDNAs encoding Hg2 and Hg3 should be cloned to allow their expression with Hg4 and Hg5. Electrophysiology on oocytes expressing combinations of these subunits should confirm whether this novel class of receptor does indeed co-assemble with GluClα and GluClβ, and if so the effect the addition of this subunit has on agonist and antagonist affinity can be studied. Subunit-specific antisera should be raised to the intracellular loop of each subunit, expressed recombinantly as a fusion protein. These antisera can then be used for IC, using the whole-worm method optimised in 5.3.1 to map the expression of the two subunits.

In the long term, *C. elegans* and *A. suum* offer significant advantages as model nematodes. The cloning work discussed in this thesis has shown that the amino acid sequences of TGIC subunits are generally well conserved across the phylum. All the evidence to date points to similarly conserved neuroanatomy, neuropeptide repertoire and classical neurotransmitter distribution (White *et al.* 1986, Holden-Dye and Walker 1994, Veglia 1915, Maule *et al.* 1996, McIntire *et al.* 1993b and Guastella *et al.* 1991) and the need for a "parasitic model" has not been demonstrated. Orthologues for 3 of the 5 *H. contortus* TGICs discussed in 1.3.3.1. have been cloned from *C. elegans*, and one other (Hg1) is implied by the sequencing project. The remaining subunit (Hg5) is probably present but as yet undiscovered. This allows the use of well developed *C. elegans* methods to map gene expression, and the exact identification of neurons. This combination of cloning and localisation should allow the production of mechanism based screening programmes, based on an understanding of the GABA and IGluR isoforms found *in*

vivo. The lessons learnt in achieving this may well give some insights into the way different TGIC isoforms are expressed and used in our own nervous system.

9. References

Amin J., Dickerson I.M. and Weiss D.S. (1994). The agonist binding-site of the gamma-aminobutyric-acid type-A channel is not formed by the extracellular cysteine loop. *Molecular Pharmacology* **45**, 317-323.

Anderson P. (1995). Mutagenesis. *Methods in Cell Biology* **48**, 31-58.

Anderson R.C. Nematode parasites of vertebrates - their development and transmission. (1992). *Cambidge University Press.*

Andrews S.J., Hole N.J.K., Munn E.A. and Rolph T.P. (1994). Vaccination of sheep against Haemonchosis with H11, a gut membrane-derived protective antigen from the adult parasite: prevention of the periparturient rise and colostral transfer of protective immunity. *International Journal for Parasitology* **25**, 839-846.

Angstadt J.D., Donmoyer J.E. and Stretton A.O.W. (1989). Retrovesicular ganglion of the nematode *ascaris*. *Journal of comparative neurology* **284**, (3), 374-388.

Arena J. P., Liu K.K., Paress P. S., Frazier G.E., Cully, D.F., Mrozik H. and Schaeffer J.M. (1995). The mechanism of action of avermectins in *C. elegans*: correlation between activation of glutamate-sensitive chloride current, membrane binding and biological activity. *Journal of Parasitology* **81** (2) 286-294.

Atkinson H.J., Isaac R.E., Harris P.D. and Sharpe C.M. (1988). FMRFamide-like immunoreactivity within the nervous stems of ther nematodes *P.redivivus, C.elegans* and *Heterodera glycines. Journal of Zoology* **216**, 663-671.

Baldwin E. and Moyle V. (1949). A contribution to the physiology and pharmacology of *Ascaris lumbricoides* from the pig. *Journal of Physiology* **4**, 145-152.

Ballivet M., Alliod C., Bertrand S. and Bertrand D. (1996). Nicotinic acetylcholine-receptors in the nematode *C. elegans*. *Journal of molecular biology* **258** (2), 261-269.

Bamber B. and Jorgensen E.M (1995). Surprise, unc-49 encodes a $GABA_A$ receptor. *Worm Breeders Gazette* wm**95** 111.

Barnard E. A. (1992). Receptor classes and the transmitter-gated ion channels. *TIBS* **17**, 368-74.

Barnard E.A. (1996). The transmitter-gated channels - a range of receptor types and structures. *TIPS* **17** (9), 305-309.

Beesley J.E. Immunocytochemistry: A practical approach (1993). *IRL press Washington.*

Bird A.F. The structure of nematodes (1971). *Academic Press. London.*

Birnboim H.C. and Doly J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513.

Brooks T.J. Essentials of medical parasitology (1963). *Macmillan.*

Brownlee D.J.A., Fairweather I., Johnston C.F. and Shaw C. (1994). Immunocytochemical demonstration of peptidergic and serotonergic components in the enteric nervous system of the roundworm, *Ascaris suum* (Nematoda, Ascaroidea). *Parasitology* **108**, 89-103.

Brownlee D.J.A., Holden-dye L., Fairweather I., Walker R.J. (1995). The action of serotonin and the nematode neuropeptide KSAYMRFamide on the pharyngeal muscle of the parasitic nematode, *A. suum. Parasitology* **111** (3), 379-384.

Brownlee D.J.A., Fairweather I., Holden-Dye L., Walker R.J. (1996). Nematode neuropeptides - localization, isolation and functions. *Parasitology Today* **12** (9), 343-351.

Catty D. Antibodies: a practical approach (1988). *IRL press Washington.*

Chen R., Belelli D., Lambert J.J., Peters J.A., Reyes A. and Lan N. C. (1994). Cloning and functional expression of a *Drosophila* γ-aminobutyric acid receptor. *PNAS* **91** 6609-73.

Chomzynski,P and Sacchi,N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. *Anal. Biochem* **162** 156-9.

Cully D.F., Vassilatis D.K., Liu K.K., Paress P.S., Van der Ploeg L.H.T., Schaeffer J.M. and Arena J.P. (1994). Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. *Nature* **371**, 707-711.

Cully D.F., Paress P.S., Liu K.K., Schaeffer J.M. and Arena J.P. (1996). Identification of a *D. melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. *Journal of Biological Chemistry* **271** (33), 20187-20191.

Davies P.A., Hanna M.C., Hales T.G. and Kirkness E.F. (1997). Insensitivity to anaesthetic agents conferred by a class of $GABA_A$ receptor subunit. *Nature* **385** (6619), 820-823

Davis R.E., and Stretton A.O.W. (1996) The motornervous system of *Ascaris*: Electrophysiology and anatomy of the neurons and their control by neuromodulators. *Parasitology* **113**, pS97-S117

Del Castillo J., De Mello W.C., and Morales T. (1963). The physiological role of acetylcholine in the neuromuscular system of *Ascaris lumbricoide*. *Arch. Physiol. Internat. Biochim.* **71**, 741-757.

Del Castillo J., De Mello W.C., and Morales T. (1964). Inhibitory action of GABA on *Ascaris* muscle. *Experientia* **20**, 141-143.

Devereux J., Haeberli P. and Smithies O. 1984. Comprehensive set of sequence-analysis programs for the vax. *Nucleic Acids Research* **12**, 387-395.

Don R.H., Cox P.T., Wainwright B.J., Baker K. and Mattick J.S. (1991). Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* **19** (14), 4008.

Duplay P., Bedouelle H., Fowler A., Zabin I., Saurin W. and Hofnung M. (1984). Sequences of the malE gene and its product, the maltose binding protein of *E. coli* K12. *Journal of Biological Chemistry* **259** (16), 10606-10613.

Eastham H., Wonnacot S., Wolstenholme A.J.W. and Lunt G. (1997). *Submitted to European Journal of Neuroscience.*

Emery D.L., McClure S.J. and Wagland B.M. (1993). Production of vaccines against gastrointestinal nematodes of livestock. *Immunology and Cell Biology* **71**, 463-472.

Emini E.A., Hughes J.V., Perlow D.S., Boger J. (1985). Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *Journal of Virology* **55**, 836-839.

Endo S. and Olsen R.W. (1992). Preparation of antibodies to beta-subunits of $GABA_A$ receptors. *Journal of Neurochemistry* **59**, 1444-1451.

Epstein H.F. and Shakes D.C. *C. elegans*: Modern biological analysis of an organism (1995). In, Methods in Cell Biology **48**, *Academic Press. London.*

Ffrench-Constant R.H., Mortlock D.P., Shaffer C.D., macIntyre R.J. and Roush R.T. (1991). Molecular cloning and transformation of cyclodiene resistance in *Drosophila*: An invertebrate γ-aminobutyric acid subtype A receptor locus. *Proc. Natl. Acad. Sci.* **88**, 7209-7213.

Ffrench-Constant R.H. and Rocheleau T.A. (1993). *Drosophila* γ-aminobutyric acid receptor gene *rdl* shows extensive alternative splicing. *J. Neurochem* **60**, 2323-2326.

Fire A. (1986). Integrative transformation of *C. elegans*. *EMBO Journal*. **5**, 2673-2880.

Fire A., Harrison S.W., and Dixon D.K (1990). A modular set of lacZ fusion vectors for studying gene expression in *C. elegans*. *Gene* **93**, 189-198.

Frohman, M. A., and Martin G.,R. (1989). Rapid Amplification of cDNA ends using nested primers. *Technique* **1**, 165-70.

Galzi J.L., Devillersthiery A., Hussy N., Bertrand S., Changeux J.P. and Bertrand D. (1992). Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic. *Nature* **359** (6395), 500-505.

Garnier J., Osguthorpe D.J. and Robson B. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *Journal of Molecular Biology* **120**, 97.

Geary T. G., Klein R. D., Vanover L., Bowman J. W. and Thompson D. P. (1992). The nervous systems of helminths as targets for drugs. *J. Parasitol.* **78** (2), 215-230.

Geary T. G., Sims S.M., Thomas E.M., Vanover L., Davis G.P., Winterrowd C.A., Klein R. D., Ho N.F.H. and Thompson D. P. (1993). *H. contortus*: Ivermectin-induced paralysis of the pharynx. *Experimental Parasitology* **77**, 88-96.

Gomezsaladin E., Wilson D.L. and Dickerson I.M. (1994). Isolation and *in situ* localisation of a cDNA encoding a KEX2-like prohormone convertase in the nematode *C. elegans*. *Cellular and Molecular Neurobiology* **14** (1), 9-25.

Grenningloh G., Gundelfinger E., Schmitt B., Betz H., Darlison M.G., Barnard E.A., Schofield P.R. and Seeburg P.H. (1987). Glycine vs GABA receptors. *Nature* **330** (6143), 25-26.

Guastella J., Johnson C.D. and Stretton A.O.W (1991). GABA-immunoreactive neurons in the nematode *Ascaris*. *Journal of Comparative Neurology* **307**, 584-597.

Guastella J., Stretton A.O.W. (1991). Distribution of H_3-GABA uptake sites in the nematode *Ascaris*. *Journal of Comparative Neurology* **307**, 598-608.

Gyenes M., Wang Q., Gibbs T.T. and Farb D.H. (1994). Phosphorylation factors control neurotransmitter and neuromodulator actions at the γ-aminobutyric acid type A receptor. *Molecular Pharmacology* **46**, 542-549.

Harlow E. and Lane D. Antibodies: a laboratory manual (1988). *Cold Spring Harbor press.*

Hart A. C., Sims S. and Kaplan J.M. (1995). Synaptic code for sensory modalities revealed by *C. elegans* GLR-1 glutamate receptor. *Nature* **378**, 82-85.

Harvey R. J., Vreugdenhill E., Zaman E., Bhandal N., Usherwood S., Barnard E. A., and Darlison M. J. (1991). Sequence of a functional invertebrate $GABA_A$ receptor subunit which can form a chimeric receptor vertebrate α subunit. *EMBO. Journal* **10**, 3239-3245.

Harvey R. J., Chinchetru M.A. and Darlison M. J. (1994). Alternative splicing of a 51-nucleotide exon that encodes a putative protein kinase C phosphorylation site generates two forms of the chicken γ-aminobutyric acid receptor β_2 subunit. *Journal of Neurochemistry* **62** (1), 10-16.

Holden-Dye L. and Walker R.J. (1988). ZAPA - a potent agonist at GABA receptors on the somatic cells of *ascaris*. *British Journal of Pharmacology* **95**, 3-5.

Holden-Dye L., Krogsgaard-Larsen P., Neilsen L. and Walker R.J. (1989) GABA receptors on the somatic muscle cells of the parasitic nematode *A. suum*. *British Journal of Pharmacology* **98**, 841-850.

Holden-Dye L. and Walker R.J (1990). Avermectin and avermectin derivatives are antagonists at the γ-aminobutyric acid (GABA) receptor on the somatic muscle-cells of *ascaris* - is this the site of anthelmintic action? *Parasitology* **101**, 265-271.

Holden-Dye L. and Walker R.J (1994). Characterisation of identifiable neurons in the head ganglia of the parasitic nematode *Ascaris suum*: a comparison with central neurons of *C. elegans*. *Parasitology* **108**, 81-87.

Holding C., and Monk M. (1990). Detection of single copy gene-sequences in a single cell by PCR amplification. *Genetical Research* **55**, 120.

Hopp T.P. and Woods K.R. (1981). Prediction of protein antigenic determinants from amino-acid sequences. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences (PNAS)* **78** (6), 3824-3828.

Innis M. A. PCR protocols: a guide to methods and applications. (1990). *Academic Press, London.*

Itakura K., Rossi J.J. and Wallace R.B. (1984). Synthesis and use of synthetic oligonucleotides. *Annual Review of Biochemistry* **53**, 323-356.

Jameson B.A. and Wolf H. (1988). The antigenic index - a novel algorithm for predicting antigenic determinants. *Computer Applications in the Biosciences* **4**, 181-186.

Jarrett E.E.E., Jarrett W.F.H., & Urquart G.M. (1968). Quantitative studies on the kinetics of establishment and expulsion of intestinal nematode populations in susceptible and immune hosts. *Nippostrongylus brasiliensis* in the rat. *Parasitology* **58**, 625-639.

Jarry D. M.J. Human Parasitology (1967). *Pergamon Press.*

Jenkins P.J. (1994) Development of antibodies against the HG1 receptor sub-unit of *Haemonchus contortus*. Project report - University of Bath. School of Biological Sciences number **570**

Johnson C. D. and Stretton A. O. W. (1985). Localisation of choline-acetyltransferase within identified motoneurons of the nematode *Ascaris*. *Journal of Neuroscience* **5**, 1984-1992.

Johnson C. D., and Stretton A. O. W. (1987). GABA-immunoreactivity in inhibitory motor neurons of the nematode *Ascaris*. *Journal of Neuroscience* **7**, 223-235.

Johnston G.A.R. (1994). $GABA_C$ receptors. *Progress in Brain Research* **100**, 61-65.

Karplus P. A. and Schultz G.E. (1985). Prediction of chain flexibility in proteins - a tool for the selection of peptide antigens. *Naturwissenschaften* **72**, 212-213.

Keating C.D., Holden-Dye L., Thorndyke M. C., Williams R.G., Mallett A. and Walker R.J. (1995). The FMRFamide-like neuropeptide AF2 is present in the parasitic nematode *H. contortus*. *Parasitology* **111**, 515-521.

Kofuji P., Wang J.B., Moss S.J., Huganir R.L. and Burt D.R. (1991). Generation of two forms of the γ-aminobutyric$_A$ receptor γ_2-subunit in mice by alternative splicing. *J. Neurochem* **56** (2), 713-715.

Kreienkamp H-J., Maeda R.K., Sine S. M. and Taylor P. (1995). Intersubunit contacts governing assembly of the mammalian nicotinic acetylcholine receptor. *Neuron* **14**, 635-644.

Kwa M.S.G., Veenstra J.G and Roos M.H. (1993). Molecular characterisation of β-tubulin genes present in benzimidazole resistant populations of *Haemonchus contortus*. *Molecular & Biochemical Parasitology* **60**, 133-144.

Kwa M. S. G., Veenstra J.G. and Roos M.H. (1994). Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype-1. *Molecular & Biochemical Parasitology* **63**, 299-303.

Kwa M.S.G., Veenstra J.G., Van Dijk M. and Roos M.H. (1995). β-Tubulin genes from the parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*. *Journal of Molecular Biology* **246**, 500-10.

Lacey E. and Gill J.H. (1994). Biochemistry of benzimidazole resistance. *Acta Tropica* **56**, 245-262.

Laughton D.L. (1993). A Characterisation of nematode neuroreceptors. *PhD Thesis University of Bath.*

Laughton D.L., Amar M., Thomas P., Towner P., Harris P., Lunt G.G. and Wolstenholme A.J. (1994). Cloning of a putative inhibitory amino acid receptor subunit from the parasitic nematode *Haemonchus contortus*. *Receptors and Channels* **2**, 1555-63.

Laughton D.L., Wheeler S.V., Lunt G.G. and Wolstenholme A.J. (1995). The beta-subunit of *C. elegans* avermectin receptor responds to glycine and is encoded by chromosome 1. *Journal of Neurochemistry* **64**, 2354-2357.

Laughton D., Turnbull J., Lunt G. and Wolstenholme A. (1997). The B-subunit of the *Caenorhabditis elegans* avermectin receptor is expressed on pm4 pharyngeal muscle cells. *Journal of Experimental Biology - In Press.*

Leach L., Trudgill D.L. and Gahan P.B. (1987). Immunocytochemical localization of neurosecretory amines and peptides in the free-living nematode *Goodeyus ulmi*. *Histochemical Journal* **19**, 471-475.

Lewis J.A., Fleming J.T., McLafferty S., Murphy H. and Wu C. (1987). The levamisole receptor, a cholinergic receptor of the nematode *C. elegans*. *Molecular Pharmacology* **31**, 185-193.

Loer C.M. and Kenyon C.J. (1993) Serotonin deficient mutants and male mating behaviour in the nematode *C. elegans*. *J Neuroscience* **13**(12), 5407-17.

Macdonald R.L. and Olsen R.W. (1994). GABA(A) receptor channels. *Annual Review of Neuroscience* **17**, 569-602

McIntire S.L., Garriga G., White J., Jacobson D. and Horvitz H.R. (1992). Genes necessary for directed axonal elongation or fasciculation in *C. elegans*. *Neuron* **8**, 307-322.

McIntire S.L., Jorgensen E. and Horvitz H.R. (1993a). Genes required for GABA function in *C. elegans*. *Nature* **364**, 334-7.

McIntire S.L., Jorgensen E., Kaplan J. and Horvitz H.R. (1993b). The GABAergic nervous system of *C. elegans*. *Nature* **364**, 337-41.

McKernan R.M., Quirk K., Prince R., Cox P.A., Gillard N.P., Ragan C.I. and Whiting P. (1991). $GABA_A$ receptor subtypes immunopurified from rat brain with alpha subunit-specific antibodies have unique pharmacological properties. *Neuron* **7**, 667-676.

Maizels R. M., Blaxter M. L. and Selkirk M. E. (1993). Forms and functions of nematode surfaces. *Exp. Parasitol.* **77**, 380-4.

Mandel M. and Higa A. (1970). Calcium-dependant bacteriophage DNA infection. *Journal of Molecular Biology* **53**, 154.

Maricq A.V., Peckol E., Driscoll M. and Bargmann C.I. (1995). Mechanosensory signalling in *C. elegans* mediated by the GLR-1 glutamate receptor. *Nature* **378**, 78-81.

Marks N.J., Shaw C., Maule A.G., Davis J.P., Halton D.W., Verhaert P., Geary T.G. and Thompson D.P. (1995). Isolation of AF2 (KHEYLRFamide) from *C. elegans*: evidence for the presence of mnore than one FMRFamide-related peptide-encoding gene. *Biochemical and Biophysical Research Communications* **217**, 845-851.

Martin R.J. (1980). The effect of GABA on the input conductance and membrane potential of *Ascaris* muscle. *British journal of pharmacology* **71**, 99-106.

Martin R.J. Electrophysiological effects of piperazine and diethylcarbamazine on *Ascaris suum* somatic muscle (1982). *British Journal of Pharmacology* **77**, 255-265.

Martin R.J. (1987). The χ-aminobutyric acid receptor of *Ascaris* as a target for anthelminthics. *Biochemical Society Transactions* **17**, 61-65.

Martin R. J. (1991). The physiology and pharmacology of neuromuscular transmission in the nematode parasite, *Ascaris suum. Parasitology* **102,** S41-58.

Martin R.J. (1993). Neuromuscular transmission in nematode parasites and anti-nematodal drug action. *Pharmacology & Therapeutics* **58,** 13-50.

Martin S.A.M., Thompson F.J. and Devaney E. (1995). The construction of spliced leader cDNA libraries from the filarial nematode *Brugia pahangi. Molecular and Biochemical Parasitology* **70,** 221-225.

Maule A.G., Shaw C., Bowman J.W., Halton D.W., Thompson D.P., Geary T.G. and Thim L. (1994). The FMRFamide-like peptide AF2 *(A.suum)* is present in the free-living nematode *P.redivivus. Parasitology* **109,** 351-356.

Maule A.G., Geary T.G., Bowman J.W., Shaw C., Halton D.W. and Thompson D.P. (1996). The pharmacology of nematode FMRFamide-related peptides. *Parasitology Today* **12,** 351-357.

Mendozadegives P. and Vazquezprats V.M. (1994). Reduction of *H. contortus* infective larvae by three nematophagous fungi in sheep fecal cultures. *Veterinary Parasitology* **55** (3), 197-203.

Mongan N.P., Baylis H.A. and Sattelle D.B. (1996). An extensive nAChR gene family in *C. elegans. Worm Breeders Gazzette* **14.3,** 44

Moss S.J., Gorrie G.H., Amato A. and Smart T.G. (1995). Modulation of $GABA_A$ receptors by tyrosine phosphorylation. *Nature* **377** (6547), 344-348.

Munn E.A., Smith T.S., Graham M., Greenwood C.A., Tavernor A.S. and Coetzee G. (1993). Vaccination of merino lambs against haemonchosis with membrane associated proteins from the adult parasite. *Parasitology* **106,** 63-66.

Nadler S.A. (1987). Biochemical and immunological systematics of some ascaridoid nematodes: genetic divergence between congeners. *Journal of Parasitology* **73,** 811-816

Nilsen T.W. (1995). *trans*-Splicing: an update. *Molecular and Biochemical Parasitology* **73,** 1-6.

Pearson W.R. and Lipman D.J. (1988). Improved tools for biological sequence comparison. *PNAS* **85** (8), 2444-2448.

Politz S.M. and Philipp M. (1992). *Caenorhabditis elegans* as a model for parasitic nematodes - a focus on the cuticle. *Parasitology Today* **8**, 6-12

Pollard S., Duggan M.J. and Stephenson F.A. (1991). Promiscuity of $GABA_A$ receptor beta-3 subunits as demonstrated by their presence in alpha-1, alpha-2 and alpha-3 subunit-containing receptor subpopulations. *FEBS Letters* **295**, 81-83.

Pribilla I., Takagi T., Langosch D., Bormann J. and Betz H (1992). The atypical m2 segment of the beta-subunit confers picrotoxinin resistance to inhibitory glycine receptor channels. *EMBO Journal* **11** (12), 4305-4311.

Price D.A. and Greenberg M.J (1977). Structure of a molluscan cardioexcitatory neuropeptide. *Science* **197**, 670-671.

Radice A.D. and Lustigman S. (1996). Cloning and characterisation of cDNAs encoding putative glutamate transporters from *C. elegans* and *O. volvulus*. *Molecular and Biochemical Parasitology* **80**, 41-53.

Reiner D.J. and Thomas J.H. (1995). Reversal of a muscle response to GABA during *C. elegans* male development. *Journal of Neuroscience* **15**, 6094-6102.

Reinitz CA and Stretton AOW (1996). Behavioral and cellular effects of serotonin on locomotion and male mating posture in *Ascaris suum* (nematoda). *Journal of Comparative Physiology A* **178**, 655-667.

Sambrook J., Fritsch E.F. and Maniatis T. Molecular cloning - a laboratory manual (1989). *2nd edition Cold Spring Harbor press.*

Sanger F., Nicklen S. and Coulson A.R. (1977). DNA sequencing with chain terminating inhibitors. *PNAS* **74**, 5463-5467.

SchmiedenV., Kuhse J. and Betz H. (1993). Mutation of a glycine receptor subunit creates a β-alanine receptor responsive to GABA. *Science* **262**, 256-2258.

Segerberg M.A. and Stretton A.O.W. (1993). Actions of cholinergic drugs in the nematode *Ascaris suum* - complex pharmacology of muscle and motor neurons. *Journal of general physiology* **101**, 271-296.

Shivers B.D., Killisch I., Sprengel R., Sontheimer H., Kohler M., Schofield P.R. and Seeburg P.H. (1989). 2 Novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. *Neuron* **3**, 327-337.

Sigel E., Baur R., Kellenberger S. and Malherbe P. (1992). Point mutations affecting antagonist affinity and agonist dependant gating of $GABA_A$ receptor channels. *EMBO Journal* **11** (6), 2017-2023.

Sithigorngul P., Stretton A.O.W., Cowden C. (1990). Neuropeptide diversity in *ascaris* - an immunocytochemical study. *Journal of Comparative Neurology* **294**, 362-376.

Smith G.B. and Olsen R.W. (1995). Functional domains of $GABA_A$ receptors. *TIPS* **16**, 163-168.

Smyth J.D. Introduction to animal parasitology (Third Edition 1994). *Cambridge University Press.*

Squire M.D, Tornoe C., Baylis H.A., Fleming J.T., Sattelle D.B. and Barnard E.A (1995). Molecular cloning and functional coexpression of a *C. elegans* nicotinic acetylcholine receptor subunit (ACR-2). *Receptors & Channels* **3**, 107-115.

Stephenson F.A. and Duggan M.J. (1990). Molecular approaches to the structure and function of the $GABA_A$ receptors. *Oxford University Press.*

Steward O. and Banker G. A. (1992). Getting the message from gene to synapse - sorting and intracellular transport of RNA in Neurons. *TINS* **15**, 180-186.

Stretton A.O.W. (1976). Anatomy and development of the somatic musculature of the nematode *Ascaris*. *Journal of Experimental Biology* **64**, 773-788.

Stretton A.O.W., Cowden C., Sithigorngul P. And Davis R.E. (1991). Neuropeptides in the nematode *Ascaris suum. Parasitology* **102**, S107-S116.

Stringfellow F. (1986). Cultivation of *H. contortus* (Nematoda, Trichostrongylidae) from infective larvae to the adult male and the egg-laying female. *Journal of Parasitology* **72**, 339-345.

Strong L., Wall R., Woolford A. and Djeddour D. (1996). The effect of faecally excreted ivermectin and fenbendazole on the insect colonisation of cattle dung following the oral administration of sustained-release boluses. *Veterinary Parasitology.* **62**, 253-266.

Swope S.L., Moss S.J., Blackstone C.D. and Huganir R.L. (1992). Phosphorylation of ligand-gated ion channels - a possible mode of synaptic plasticity. *FASEB Journal* **6**, 2514-2523.

Taylor M.A., Hunt K.R. and Wilson C.A. (1991). Effectiveness of clean grazing strategies in controlling *H. contortus* infections in sheep in the United Kingdom. *Veterinary Record* **129**, 166-170.

Thomas J.H. (1994). The mind of a worm. *Science* **264**, 1698-9.

Thompson M., Shotkoski F., Ffrench-Constant R. (1993). Cloning and sequencing of the cyclodiene insecticide resistance gene from the yellow fever mosquito *Aedes aegypti. FEBS* **325** (3), 187-90.

Towner P. and Gärtner W. (1992) cDNA cloning of 5' terminal regions. *Nucleic Acids Research* **20**, 4669-4670.

Trent C., Tsung N. and Horvitz H.R. (1983). Egg-laying defective mutants of the nematode *C. elegans. Genetics* **104**, 619-647.

Unwin N. (1995). Acetylcholine receptor channel imaged in the open state. *Nature* **373,** 37-43.

Veglia F. (1915). The anatomy and life history of *H. contortus. Third and fourth reports of the Director of Veterinary Research, Department of Agriculture, Union of South Africa.*

Wafford K.A., Burnett D.M., Leidenheimer N.J., Burt D.R., Wang J.B., Dunwiddie T.V., Harris R.A., Sikela J.M. and Kofuji P. (1991). Ethanol sensitivity of the$GABA_A$ receptor expressed in *Xenopus* oocytes requires 8 amino-acids contained in the gamma-2l subunit. *Neuron* **7**, 27-33.

Wafford K.A. and Whiting P.J. (1992). Ethanol potentiation of $GABA_A$ receptors requires phosphorylation of the alternatively spliced variant of the $\gamma 2$ subunit. *FEBS Letters* **313** (2), 113-7.

Walker R.J. and Holden-Dye, L. (1991). Evolutionary aspects of transmitter molecules, their receptors and channels. *Parasitology* **102**, S7-S29.

Walker R.J. Holden-Dye, L. and Franks C.J. (1993). Physiological and pharmacological studies on annelid and nematode body wall muscle. *Comp Biochem Physiol* **106C**, (1), 49-58.

Waller P.J. (1992). Prospects for biological control of nematode parasites of ruminants. *New Zealand Veterinary Journal* **40**, 1-3.

Weinshenker D., Garriga G., Thomas J.H. (1995). Genetic and pharmacological analysis of neurotransmitters controlling egg-laying in *C. elegans*. *Journal of Neuroscience* **15** (10), 6975-6985.

Wheeler J.E., Kendall S.J., Butters J., Hollomon D.W. And Hall L. (1995). Using allele-specific oligonucleotide probes to characterize benzimidazole resistance in *Rhynchosporium secalis*. *Pesticide Science* **43**, 201-209.

White J.G., Southgate E., Thomson J.N. and Brenner S. (1986). The structure of the nervous system of the nematode *C. elegans*. *Philosophical Transactions Of The Royal Society Of London Series B- Biological Sciences* **314** (1165), 1-340.

World Health Organisation annual report (1996). Homepage http://www.who.org

Xu M. And Akabas M.H. (1996). Identification of channel-lining residues in the M2 membrane-spanning segment of the $GABA_A$ receptor alpha(1) subunit. *Journal of General Physiology* **107**, 195-205.

Zaman S. H.,Harvey R.J.,Barnard E.A. and Darlison M. G. Unusual effects of Benzodiazepines and cyclodiene insecticides on an expressed invertebrate $GABA_A$ receptor (1992). *FEBS* **307** (3), 351-4.

Zhen M.,Schein J.E.,Baillie D.L. and Candido E.P.M. (1996). An essential ubiquitin-conjugating enzyme with tissue and developmental specificity in the nematode *C. elegans*. *EMBO Journal* **15** (13), 3229-3237.

10. Appendix 1.

10.1 Sequence alignment of putative inhibitory amino acid receptors from C. elegans and H. contortus.

The predicted amino acid sequences of inhibitory TGIC subunits from *C. elegans* and *H. contortus* were aligned using the GCG program PILEUP (Devereux *et al.* 1984). The two cysteine residues of the 'cys'-loop are in bold, the approximate positions of the predicted transmembrane domains are underlined.

```
          1                                                  50
 t21f21   .......... .......... .......... .......... ..........
f09c121   .......... .......... .......... .......... ..........
 c53d63   .......... .......... .....MIFTL LS........ ..........
 k10d61   ......MLQI FLINISISFP LVIFSFIFQL LSMTLFLPNW CSGFYYHDDV
f55d105   .......... .......... .......... .......... MGPIFDRANT
 f58g64   .......... .......... .......... .......... ..........
 f47a41   .......MGR CLQFGRLRVG RLRACATKTK TSSANIDATI IRHTKHQDHQ
 unc49c   .......... .......... .......... .......... ..........
 unc49a   .......... .......... .......... .......... ..........
 f11h82   .......... .......... .......... .......... ..........
    hg4   .......... ....MSQYMM VAVAAVVAVA GS........ ......SQIS
 gluclβ   .......... .MTTPSSFSI LLLLLLMPVV TN........ ......GEYS
 Cegbr3   .......... ....MWHYRL TTILLIISII HS........ ......IRAK
 Cegbr2   .......... ....MWHYRL TTILLIISII HS........ ......IRAK
 gluclα   .......... .....MATWI VGKLIIASLI LG........ ......IQAQ
 c27h58   ........MF SSVFNFQQFL FGILIRLCLF VDLDDLHNPS TDHLPLVPSR
    hg1   .......... .......... .......... .......... ..........
c39b102   .......... .......... .......... .......... ..........
 c45b24   .......... .......... .......... .......... ..........
 c09g51   .......... .......... .......... .......... ..........
t20b129   .......... .......... .......... .......... ..........
 t24d81   .......... .......... .......... .......... ..........
```

```
          51                                                  100
 t21f21   .......... .......... .......... .......... ..........
f09c121   MLHDVIYMLL ISTVAKEVKT ESLVDPAHCE YNSNHRKSIL TVSFNLNSFL
 c53d63   .......... .......TLP VLIITTELDY SELVHSAELV SSSSYIHHKT
 k10d61   MLSSQMHNRK TPNSF.FIFL ILLSILQINI CEIIIDSDYN VSSDDDPTTT
f55d105   WGGGPTHPSN GPNTFTLCEG FFVCVSENKF FPLPPTTPF. .PTNAVAATD
 f58g64   .......... .......... .......... .......... ..........
 f47a41   DSKVTTKTSF PPPPRSFTML STLFLLLYAT RETVNAMTAE IS....PTVS
 unc49c   ........MA RPFTLIVLLS AHL..... ..LDSHINTQ L.........
 unc49a   ........MA RPFTLIVLLS AHLCLHVVVT QDEDSHINTQ L.........
 f11h82   .......... ...MSLVFIS SLLFRMGESN ENLEN..... ..........
    hg4   RRSTGGTQE. .......... .....QEILN E......... ..........
 gluclβ   MQS.....E. .......... .....QEILN A......... ..........
 Cegbr3   R.K...LKE. .......... .....QEIIQ .......... ..........
 Cegbr2   R.K...LKE. .......... .....QEIIQ .......... ..........
 gluclα   QAR...TKS. .......... .....QDIFE DDNDNGTTTL ESLARLTSPI
 c27h58   RESARNAKET VYHRLFFVMK AQLYVSVLLA LLVSSTAKKS KTKSCKRTAF
    hg1   .......... .......... .....MNLFF VFVILRTSSA LQGGIPRNER
c39b102   .......... .......... .......... .......... ..MKKYWSTL
 c45b24   .......... .......... .......... .......... ..........
 c09g51   .......... .......... .......... .......... ..........
t20b129   .......... .......... .......... .......... ......MGFF
 t24d81   .......... .......... .......... .......... ..........

          101                                                 150
 t21f21   .......... .......... .......... ......MYLQ MKDHVIVELT
f09c121   YALGLGGTHT FIFAQLFKKL ICNEFHDLLQ DYDKTMVPSN NSVQVSVELT
 c53d63   NKKPDNCTRD TDIIDRLLNG TGYNKFRIPQ .........E EGMTVVVEIW
 k10d61   TQKPRNCTRQ PAIIDKLLNG TGYNKFRIPN .........E NGVPVQVEFW
f55d105   EDGEELCTSD KTIIEKLLN. .NYKSFRTP. .........SE SGVIVWIEVW
 f58g64   ....MHYSKI ATIKFVFRTL TELKLFFFQH YI......PS HPTHVRVDMW
 f47a41   YLLDRGCAND TEIIDHLLID RAL..YYNKH KL......PS PQVDVRIEMW
 unc49c   .........L SSVLDRLTNR TTYDKRLRPR .....YGEKP VDVGIT..IH
 unc49a   .........L SSVLDRLTNR TTYDKRLRPR .....YGEKP VDVGIT..IH
 f11h82   ........NV TRLLDKLI.. ASHDRRIRPN .....YGGPP IEVNITA..H
    hg4   .......... ........LL SNYDMRVRPP PSN..YSDPM GPVTVRVNIM
 gluclβ   .......... ........LL KNYDMRVRPP PAN...SSTE GAVNVRVNIM
 Cegbr3   .......... ..RIL..... KDYDWRVRPR GMNATWPDTG GPVLVTVNIY
 Cegbr2   .......... ..RIL..... KDYDWRVRPR GMNATWPDTG GPVLVTVNIY
 gluclα   HIPIEQPQTS DSKILAHLFT SGYDFRVRPP T......DNG GPVVVSVNML
 c27h58   SRHTTNYQAW REQMTVCDLL QDYDAAVRPS GRTP.YNDTR GAVMVTTSLN
    hg1   STYNTTLISY TTKLLNTILL NQ.DKKFRPT NPD......S SPLNVEIDIS
c39b102   HLFQVLCIFI FIRTSSPLTQ PNIYNLLQQS NTRPPTANAS VPLGVKLGMY
 c45b24   .......... .......... ......MQEC LKCLIFRHFE EVTVVELAMY
 c09g51   .......... .......... .......MGD VRSAPLRAYA MPSSIGTCKN
t20b129   FEYLEFNSRI NLGKLIDTLL TDYDTHLLPE .........A EGVNVTIELH
 t24d81   .......... .......... .......... .......... ..........
```

```
          151                                                  200
 t21f21   VQSITEISEF SS.SFKADVW FSQIWRDPRL DFSD....RN YCLKNISL..
f09c121   VQDISSISEI SS.SFIADVW FSQVWYDPRL EYRN....IS .CKTNLSL..
 c53d63   IQAITSIDEL TN.DFDMDIY ITETWLDPAL NFQT....MT PCKGNLSL..
 k10d61   VQAITSINEI TN.DFEMDIY INEMWLDPAL KFDH....LN PCKQNLSV..
f55d105   VQEVNSVNEI TS.DFDMDIY VTELWMDSAL RYEH....LN PCKYNLSL..
 f58g64   VQEVTSVSEL TQ.DFEIDLY INEFWEDPAL VYED....MN PCKRNISF..
 f47a41   VQEVTSVSEL TQ.DFEIDLY MNEYWTDPGL AYDI....LN PCQGNLSF..
 unc49c   VSSISAVSEV D.MDFTLDFY MRQTWQDPRL AFGSLDLGLS KEI.DSLTVG
 unc49a   VSSISAVSEV D.MDFTLDFY MRQTWQDPRL AFGSLDLGLS KEI.DSLTVG
 f11h82   VTTISAVSEV S.M..... .......... .......... SDVEDSLTIG
    hg4   IRMLSKIDVV NEMEYSMQLT FRGQWLDSRL AYAHL...GY HNPPKFLTVP
 gluclβ   IRMLSKIDVV N.MEYSIQLT FREQWIDPRL AYENL...GF YNPPAFLTVP
 Cegbr3   LRSISKIDDV N.MEYSAQFT FREEWTDQRL AYERYEESGD TEVPPFVVLA
 Cegbr2   LRSISKIDDV N.MEYSAQFT FREEWTDQRL AYERYEESGD TEVPPFVVLA
 gluclα   LRTISKIDVV N.MEYSAQLT LRESWIDKRL SYGVKGDGQ. ...PDFVILT
 c27h58   IRSISAVSEK N.MEFVAQFR FRQEWYDDRL RFIEHQGLLS SDYRNFEFIH
    hg1   VRSMGPISEQ D.MEFSLDCY FRQKWLDRRL AFTPINPDKP VMPLASKML.
c39b102   LESLGNFRSS E.MSFDVDLY VYMSWQDPRL AHNFSDYVLI NN........
 c45b24   IEGMSSFRTQ T.MDFQLDVY FQQFWRDPRL AHNETRRVLV KD........
 c09g51   QGSTFKPTKL E.IDFQVDIY FQEKWVDHRL QHNNTKRILV KD........
t20b129   VQGVSGISEI TG.DFSLDVM YSEIWQDPRL SFKH....LN VCATNITL..
 t24d81   .......... ..MDYHLDVY FQEEWYDHRL AHNASAPILV RD........

          201                                                  250
 t21f21   ..A.SHKLPT MWSPNV.... ....EIHASP SQNILLLIFP NGTVWLNFRV
f09c121   ..D.SYVSER LWTPNVCFVN SKSTQVHKSP ASNILLIIYP NGTVWLNYRV
 c53d63   ..N.HQVLDR LWTPNSCFIN SKVAQIHNSP FRSVFLMLFP NGTVMVNYRV
 k10d61   ..S.HQVLEQ LWTPNSCFVN SKFAEIHDSP FKNVFLMIYP NGTVWVNYRV
f55d105   ..N.SEILDQ IWKPNTVFIN SKSANIHKSP FKNVFLMIYP NGTVWVNYRV
 f58g64   ..D.DKVLQR LWLPNTCFIN SKSAAIHESP FKNVFLMVFS NGTLWTNYRM
 f47a41   ..D.WAVMQN IWTPNTCFIN SKKAQLHSSP FTNVFLMVFP NGSVWSNWRI
 unc49c   V....DYLDR LKWPDTFFPN EKKSFFHLAT THNSFLRIEG DGTVYTSQRL
 unc49a   V....DYLDR LKWPDTFFPN EKKSFFHLAT THNSFLRIEG DGTVYTSQRL
 f11h82   I....DMVKT IWTPDTFFPN EKKSFFHEAT SHNSFLRIDN HGNVLRSIRL
    hg4   .....HIKSN LWIPDTFFPT EKAAHRHLID TDNMFLRIHP DGKVLYSSRI
 gluclβ   .....HVKKS LWIPDTFFPT EKAAHRHLID MENMFLRIYP DGKILYSSRI
 Cegbr3   TSENADQSQQ IWMPDTFFQN EKEARRHLID KPNVLIRIHK NGQILYSVRL
 Cegbr2   TSENADQSQQ IWMPDTFFQN EKEARRHLID KPNVLIRIHK NGQILYSVRL
 gluclα   V......GHQ IWMPDTFFPN EKQAYKHTID KPNVLIRIHN DGTVLYSVRI
 c27h58   VA....RDQS LWIPDTFFQN EKNGWYHMLN QENRFLKIRS DGKLIYDRRL
    hg1   ........KD IWVPDTYIRN GRKSYLHTLT VPNILFRVRS DGQVHVSQRL
c39b102   ....DEIRKQ IWLPDLYFAN ARQASFQEVT VPNFNLFVAP DGTVAYSCRC
 c45b24   ....KAVLHK MWKPDVYFAN ARIAEFHEVT QPNFLVWIQP DGSILYDTRI
 c09g51   ....PKLFGL LWHPDLYFAN ARTASFHDVT QPNFLVWIYP NGTVWYDCRI
t20b129   ..KVSDFRKK IWTPDTCIIN SKSSSIHSSP SENTFVILYE NGLVWSNFRL
 t24d81   L....TVFKM MWHPDVYFAN ARSAAFQDIT DDNFLVWVYP NGRVWYDARI
```

```
          251                                                      300
 t21f21   SLTGPCKLDL TYFPMDRQSC NLVFEVSMSI PSS..LQSYS YNTAEVRIVW
f09c121   QVSAPCSFEL SRFPIDAQEC HLVFE..... .......SYS YNIAEVRLNW
 c53d63   RVKGPCSLDL SNFPLDLQKC SLIYE..... .......SFN YNRQEVEMRW
 k10d61   NVKGPCDLSL ELFPLDIQEC HLIYE..... .......SFN YNNQEVRMRW
f55d105   QVKGPCSMDF SAFPMDQQSC HLTLE..... .......SFS YNNQEVDMQW
 f58g64   KLTGPCDMKL KRFPFDKQKC YLTFE..... .......SFN YNTGEVRMQW
 f47a41   KSTGPCVMDL TKFPMDSIEC TLTFE..... .......SFN YNKDEVFMRW
 unc49c   TVTATCPMDL KLFPMDSQHC KLEIES.... .........YG YSILDIMYVS
 unc49a   TVTATCPMDL KLFPMDSQHC KLEIESCKSL CSPDIFSPYG YSILDIMYVS
 f11h82   TVTANCPMSL HTFPLDRQEC ALEVE..... .......SYG YSTKDIIYHW
    hg4   SITSSCHMQL QLYPLDLQFC DFDLV..... .......SYA HTMKDIMYEW
 gluclβ   SLTSSCPMRL QLYPLDYQSC NFDLV..... .......SYA HTMNDIMYEW
 Cegbr3   SLVLSCPMSL EFYPLDRQNC LIDLA..... .......SYA YTTQDIKYEW
 Cegbr2   SLVLSCPMSL EFYPLDRQNC LIDLA..... .......SYA YTTQDIKYEW
 gluclα   SLVLSCPMYL QYYPMDVQQC SIDLA..... .......SYA YTTKDIEYLW
 c27h58   TLHLACSMHL SRYPMDHQNC EIAFA..... .......SYA YTTADIEYIW
    hg1   TIKTKCLMFL KKFPMDVQAC PIEIG..... .......SLG YFSNDVVYYW
c39b102   TLTVACSLNL RYYPMDQQLC SIRVL..... .......SYA YIAKQVNVTW
 c45b24   SMVVVCTLNL EKWPLDSQRC HLRIL..... .......SYA YTTEQLVIKW
 c09g51   SLTVLCMQDL ARYPLDSQNC GLRIL..... .......SYA YDEEQLIIRW
t20b129   NVKTPCSVNL KMFPFDSLSC EIVLE..... .......SYS FNTDEVRLMW
 t24d81   SIVSSCNMDL WKYPLDSQEC ALRIL..... .......SYA YPMTVLRLLW
               *                *

          301                                                      350
 t21f21   RDWDAV.TIP DPDSKNLPDF ELVNI.EHMN ATLVYT.... .....AGLWD
f09c121   QQWAPV.TMP PPEDFRLPDF QFYNV.TWGK TSNEYT.... .....AGMWD
 c53d63   SD.AEH.PVF NLSKIMLPDF DLFEIQT.ER RQEPYP.... .....AGMWD
 k10d61   NEKSAE.PVS VTNKIRLPDF ELIKIES.TR ISAPYP.... .....AGMWD
f55d105   MNWTTP.LSL LKKEIVLPDF VMSNYST.SL KHEIYP.... .....AGVWN
 f58g64   NQ..PY.PVI LLKRIELPDF KLVNFSV.IA VEQMYP.... .....AGWWD
 f47a41   GD...P.PLT IFKPIELPDF TMTNFST.SN KFQLYA.... .....AGYWS
 unc49c   HEKKSVST.. ..ESYELPQF VLQSI.KVVN HTQKL..... ...LSSGTYS
 unc49a   HEKKSVST.. ..ESYELPQF VLQSI.KVVN HTQK...... ...LSSGEYS
 f11h82   HGTNAV.TID E..NVHLAHF TIGEH.KHIE RTISLS.... .....TGNYS
    hg4   DPSTPV.QLK PGVGSDLPNF QLTNITTNDD CTSHTN.... .....TGSYA
 gluclβ   DPSTPV.QLK PGVGSDLPNF ILKNYTTNAD CTSHTN.... .....TGSYG
 Cegbr3   KEKKPI.QQK DGLRQSLPSF ELQDVVT.DY CTSLTN.... .....TGEYS
 Cegbr2   KEKKPI.QQK DGLRQSLPSF ELQDVVT.DY CTSLTN.... .....TGEYS
 gluclα   KEHSPL.QLK VGLSSSLPSF QLTNTST.TY CTSVTN.... .....TGIYS
 c27h58   .DVPAI.QIH EGANGALPNF EIASF.KNAS CTSKTN.... .....TGTYS
    hg1   KDV....ELD AKMGNMLSQY KILGLFKSEH NFS....... DHREIGRNIS
c39b102   FDKN...PVR FNEEIGLPEF QIEHV.SNAY CNGSYQYALT ENSYKSDDFS
 c45b24   KEDE...PIT RNPNIAMSDM HIVDL.YPGL CDGNYS.... .....TGTWS
 c09g51   NGGN...PVE VNRGIRMPDM HLKHI.KFYT KRDKYA.... .....TGIWS
t20b129   HD...V.PIT MMEKVELPDF DLIGWSTDHQ RLE.YP.... .....NGIWD
 t24d81   SEKEDVPAID RNPDITMPDM SLKHIRT.GY CNGTYA.... .....TGEWS
```

```
          351                                                    400
 t21f21   QLEVKFT... ..FRRLYGYY V..... .....LQA YMPTYLSVFI
f09c121   QLKVTFSQTF YRFKRLYGYY V..... .....LQM YLPTYLSVFI
 c53d63   ELHVTII... ..FERRFIWY F..... .....MQA YLPTYLTIFI
 k10d61   ELHVKLV... ..FERRYIWY F..... .....MQA YLPTYLTIFI
f55d105   ELTMTFV... ..FSRRYGWY Y..... .....TNM LLFFKHASPF
 f58g64   ELTVAFV... ..FERRYGWY V..... .....LQG YIPTMVTIVI
 f47a41   ELNVNFL... ..FRRRSGWY L..... .....LQG YIPTYMTVFI
 unc49c   RLRVSFI... ..FDRDSGFY FF..... .....LQI FFPA......
 unc49a   RLCWFFL... ..FKRNIGFY .I..... .....IQI YLPSVLIVVI
 f11h82   RLTAYFL... ..FKRNIGFY LIQPLKYDFK KTEKILAFQI YFPSSLIVVI
    hg4   CLRMQLT... ..LKRQFSYY L..... .....VQL YGPTTMIVIV
 gluclβ   CLRMQLL... ..FKRQFSYY L..... .....VQL YAPTTMIVIV
 Cegbr3   CARVVLR... ..LRREYSYY L..... .....IQL YIPCIMLVVV
 Cegbr2   CLRTRMV... ..LRREFSYY L..... .....LQL YIPSFMLVIV
 gluclα   CLRTTIQ... ..LKREFSFY L..... .....LQL YIPSCMLVIV
 c27h58   CLKVEIR... ..LNRVFSFF L..... .....LQL YIPSSMLVGV
    hg1   VLKVYFK... ..LQRQQGYY V..... .....LQI YTPCTLLVVM
c39b102   CLSGNLF... ..LSRSIGYN L..... .....VQS YIPTGLIVMI
 c45b24   CVTAEFF... ..VKREITHH V..... .....MQS YVPTTLIVVI
 c09g51   SAVAEFH... ..VDREITHH I..... .....IQS YIPTSLIVII
t20b129   RAKVKFT... ..FARRYGFY L..... .....FQS YFPTSLTVIS
 t24d81   CMTAIFY... ..VEREMMHH V..... .....MQT YVPTALIVVI
                                                        TM1

          401                                                    450
 t21f21   SWIAFWIDTK ALPARITLGV SSLMALTFQF GNIVK.NLPR VSYVKALDIW
f09c121   SWIAFWIDTR ALPARITLGV SSLMALTFQF GNIVK.NLPR VSFVKAIDLW
 c53d63   SWISFSLGSR AIPARTMLGV NSLLAIVFSF GNIMR.NLPR VSYIKGIDVW
 k10d61   SWISFSLGTK AMPARTMLGV NALLAMIFQF GNIMR.NLPR VSYVKAIDVW
f55d105   SWISFCLGPK MIPARTMLGV NSLLALTFQF GNIMR.NLPR VSYVKALDVW
 f58g64   SWISFYLGPR AIPARTMLGV NSLLAMTFQF GNIIR.NLPR VSYVKAIDVW
 f47a41   SWIPFYLGPK AIPARTMIGV NALLALTFQF GNIIR.NLPR VSYIKAIDVW
 unc49c   SLISFWINRD SAPSRTLIGT MTVLTETHLM TG.TNRRLPP VAYVKAVDVF
 unc49a   SWVSFWLSRD ATPARVALGV TTVLTMTTLM TTMTNSSMPK VSYVKSIDIF
 f11h82   SWVSFWLNRE AVQARVAIGV TTVLTMTTLM TS.TNASLPK VSYVKSLDVF
    hg4   SWVSFWIDMH STAGRVALGV TTLLTMTTMQ AAI.NAKLPP VSYVKVVDVW
 gluclβ   SWVSFWIDLH STAGRVALGV TTLLTMTTMQ SAI.NAKLPP VSYVKVVDVW
 Cegbr3   SWVSFWLDKD AVPARVSLGV TTLLTMTTQA SGI.NTKLPP VSYIKAVDVW
 Cegbr2   SWVSFWLDKD SVPARVTLGV TTLLTMTTQS SGI.NANVPP VSYTKAIDVW
 gluclα   SWVSFWFDRT AIPARVTLGV TTLLTMTAQS AGI.NSQLPP VSYIKAIDVW
 c27h58   AWVSYWIDWK STAARVPLAI VTLLTMITTS HAI.NSNLPP VSYAKSIDIW
    hg1   SWVSFWINKE ASPARVALGI MTVLSMSTLG FGL.RTDLPK VSHSTALDIY
c39b102   SWVSFWIDRR AVPARVTLSF TTLVSLTTLG NGL.RFGLPQ VSYAKAIDLW
 c45b24   S..CFWLDVE AVPARVSLAI TTLLTLSTQA NA.ARMALPE VSYMKAIDVW
 c09g51   SWFSFWLDVE AVPGRVSLSI TTLLTLATQS SA.ARMALPQ ASDVKAIDVW
t20b129   SWVGFFFDVR SVSARITLGV SSLLALTFQF GNVLR.HLPR VSYIKCLDVW
 t24d81   SWFNFWLEID SAPARVSLSI TTLLTISTQA NAV.KLALPE VSYMKAIDVW
          TM1                  TM2                        TM3
```

```
          451                                                        500
 t21f21   MF..... ..... ..... ....GCVGFI FLSLVELAVV
f09c121   FF..... ..... ..... ....VCVAFI FFSLVELAVV
 c53d63   ML..... ..... ..... ....VSMTFI FCSLLELAIV
 k10d61   ML..... ..... ..... ....VSMTFI FLSLLELAIV
f55d105   ML..... ..... ..... ....VCLTFV FCSLLELAII
 f58g64   ML..... ..... ..... ....SGMLFI FLSLLELAVV
 f47a41   MLRFVSVSTS SLLVLPLQLF ITSAPKNLHI FFSFSGICFV FASLFELATI
 unc49c   L..... ....GFCYLL VILAYVKAVD VFLGFCYLLV ILALIEYACV
 unc49a   L..... ....GVCFMM V..... ....FC.... ..SLLEYAAV
 f11h82   L..... ..... ..... ...GVCFFIV FASLLEYAAI
    hg4   L..... ..... ..... ...GACQTFV FGALLEYAFV
 gluc1β   L..... ..... ..... ...GACQTFV FGALLEYAFV
 Cegbr3   I..... ..... ..... ...GVCLAFI FGALLEYAVV
 Cegbr2   I..... ..... ..... ...GVCLAFI FGALLEFALV
 gluc1α   I..... ..... ..... ...GACMTFI FCALLEFALV
 c27h58   V..... ..... ..... ...GACVVFI FFSLIEYAVV
    hg1   IL..... ..... ..... ....SCFGFV FAAMVEYSVI
c39b102   Y..... ..... ..... ...GACMFFV FCALLEFATI
 c45b24   M..... ..... ..... ...GACMMFV FGVMIEFTIV
 c09g51   M..... ..... ..... ...GTCMAFV FSAMIEFTVV
t20b129   MI..... ..... ..... ....FSVIFI FCTLVELAIV
 t24d81   M..... ..... ..... ...GSCMAFV FGVMIEFTIC
                                                  TM3

          501                                                        550
 t21f21   G.FADKLEAK RRRHNRCKEQ LMM..... .R...SDSEQ QWLSRLSG..
f09c121   G.FVDKITEI KRRSRRIKFQ RAIAGGTIKN DR...PVSFR KYSCTSKC..
 c53d63   G.FMVRDETV AKKK.QQKKI SGN......I SR...EESPH ..........
 k10d61   G.YKTKNEEG SKKKCPHKKL LDN......F .....EASPA ..........
f55d105   G.SMGARSE. .NRQAQQQKQ QDEEATKHQK GR...ENSTC SHLMSPSSCP
 f58g64   G.FMSRNEGL PPKVKKRKRQ EDDDEGFSWK SM...QTSPH LELRQFW...
 f47a41   G.FLMRNEGK PATKSSRSTK NSNRWQQPSV CR...EDTQR KSQNIVFCER
 unc49c   A.Y.SKKKNE DRRRREKKTE HKPAP..... .......... ..........
 unc49a   G.YISKRMKL VRARKESRSM LTPLPHLESL PPKRTLSVPS YFNNTTYRPF
 f11h82   G.Y..LMKRN RSVPAASPVQ YYETEEFFEI Q......CHP KFVRG..AIN
    hg4   S.YQDSQRQT EQAKSRE... .......... .......... ..........
 gluc1β   S.YQDSVRQN D..RSRE... .......... .......... ..........
 Cegbr3   N.YYGRKE.F LRKEKKK... .......... .......... ..........
 Cegbr2   N.YAARKD.M TQ..... .......... .......... ..........
 gluc1α   N.HIANKQGV ERKARTE... .......... .......... ..........
 c27h58   N.YVGILDEH RQMKKAA... .CNRSRLSNV I......END NFGESLQSLT
    hg1   N.YAQIVYIR KQVHDLK... .......... ....GLESNR AMEKMRLFTA
c39b102   NSYMRKSEKF DSMAKKMQSV VLTGRTRDYL VRGIKESMRI AGGAAGNVVD
 c45b24   NYAQRQGRKW H....RTDAE IVMASSLESV KSGNAEKKPS RGDLGAHARH
 c09g51   NYCVRR.KVR T....KIKPR .GLSEQVHDM VAQYREKKDK FNN..GNCEI
t20b129   CQLNRWERER QIGSKVLGHW LNQIRKTRKK ESKADEGGGG GVGGLLRKRI
 t24d81   HYAKNLEMLR G....DGQPS LIVDTALSTL FGAARDIDDL VRKVATLATP
```

```
          551                                                        600
 t21f21   .......... ........QR PQVSETNSDA THTIQI.NDG NGNVRRRKSE
f09c121   .......... ........NS VRYNINNNDD EELYNISGET NGNGVSWKNN
 c53d63   .......... .......... .......... ...GIIS.ER RFMFPPGCSE
 k10d61   .......... .......... .......... ...GLCRYEK RFMLPVERRS
f55d105   NSPRICRNHI PNDVPQSFKS YGSTGINEGK MENTLISGNG CYPRMRKRLI
 f58g64   .......... ...VDKRVNS LRNDSAVPPV EDYAPMELEQ PYQNITKRRE
 f47a41   LDNTSRLMFP GLYVCGS... .......... .......... ..........
 unc49c   .......... .......... .......PTP DILHEMLDWD VRLAEC.TCN
 unc49a   YSST..DQTS NLYIPESQRT TIFSNEDAVP NELTPMLGRS NSQASVFLYQ
 f11h82   YCDGVLKREA SR..KKSRNR RDSSMKNYQN NNIHHRPSDG HVDQRIPLLS
    hg4   ..KAARKAQK RRAKMELVER EQYQPPCTCH LYQDYER..S FRDRLRRYFT
 gluclβ   ..KAARKAQR RREKLEMVDA EVYQPPCTCH TFEARE...T FRDKVRRYFT
 Cegbr3   ..KTRIDDCV CPSDRPPLRL DL....SAYR SVK....RLP IIKRISEILS
 Cegbr2   .........V SQRIRQMKQL PT....EGYR PLSASQGRSS FCCRI..FVR
 gluclα   ..REKAEIPL LQNLHNDVPT KVFNQEEKVR TVPLNRRQMN SFLNLLETKT
 c27h58   FSPQEKKRLI RRRPKKNMEM QEGDFEAIEM VDRGPPRSAG LMEEGWTFHD
    hg1   GLMGARRETI QVDDLVLDEE EDLPWYSCFF RKKTKKQSSM FYRMAVKAAK
c39b102   ALNDDWCTYS TENGRKSFS. .......AMV GDIFERAGNA ISPRRNRYRG
 c45b24   LF.LRLRTNE RQHLVEDP.. ...PEQIAMN DTAYDTVSQA YSNS.FSDEE
 c09g51   SYEMALQPNE DNATVQRNFE KKEVREMNQA SLFVRRSLLP TSKR.KTIED
t20b129   PVLAQLKAAA TDSNSGAATA MTTAIQPP.N TNLNSITNSD NSKLVANNFT
 t24d81   TIGLPRRRHS RPPAPHHAPH SRHPHTIALL KFCFAITKHH RSAHYTHKL.

          601                                                        650
 t21f21   EKRQKLLLQM ESPMFVN... .......... .......... ..........
f09c121   SSRE...... ASADM..... .......... .......... ..........
 c53d63   SSKS...... LSSCTSG... .......... .......... ........W.
 k10d61   ARWGGIIRFQ VFQDFWN... .......... .......... ........W.
f55d105   IASSSTISHA PNANRSEKVL LLDGLEETQF SQVETKFSSM ASIKMKKHW.
 f58g64   KRKWMSGLRK KWRAMRE... .......... .......... ........L.
 f47a41   .......... .......... .......... .......... ..........
 unc49c   AAPTSIIAVI KQSNRFCVSH .......... .......... ..........
 unc49a   TAVISDDEFG RFWRWL..RP .......... .......... ..........
 f11h82   MVPMPPIPFK PQ........ .......... .......... ..........
    hg4   KPDYL..... .......... .......... .......... ..........
 gluclβ   KPDYL..... .......... .......... .......... ..........
 Cegbr3   TNIDI..... .......... .......... .......... ..........
 Cegbr2   RYKER..... .......... .......... .......... ..........
 gluclα   EWNDI..... .......... .......... .......... ..........
 c27h58   TTDLVYIGQR KRVE...... .......... .......... ..........
    hg1   ARRKLKMKDP AKV....... .......... .......... ..........
c39b102   VDQCGHFLLI TLKSVFWF.. .......... .......... ..........
 c45b24   AAPVVQPPQN EDAEAQEEVW AEQKETNLSE VWKESDPAER LAHWNEKEND
 c09g51   RINRVE.... .......... .......... .......... ..........
t20b129   SIEHETYAYE KKRGFSHCFQ RF........ ......VYAI CPPDRDWTI.
 t24d81   .......... .......... .......... .......... ..........
```

```
          651                                                       700
 t21f21   .......... .......... .......... .......... ..........
f09c121   .......... .......... .......... .......... ..........
 c53d63   .......... .......... .......... .......... ..........
 k10d61   .......... .......... .......... .......... ..........
f55d105   .......... .......... .......... .......... ..........
 f58g64   .......... .......... .......... .......... ..........
 f47a41   .......... .......... .......... .......... ..........
 unc49c   .......... .......... .......... .......... ..........
 unc49a   .......... .......... .......... .......... ..........
 f11h82   .......... .......... .......... .......... ..........
    hg4   .......... .......... .......... .......... ..........
 gluclβ   .......... .......... .......... .......... ..........
 Cegbr3   .......... .......... .......... .......... ..........
 Cegbr2   .......... .......... .......... .......... ..........
 gluclα   .......... .......... .......... .......... ..........
 c27h58   .......... .......... .......... .......... .LVRWCSVLS
    hg1   .......... .......... .......... .......... ..........
c39b102   .......... .......... .......... .......... ..........
 c45b24   ASSPLMGNGR AHVRYGAAEK MKNRKCNDSS RKKEKWSSAI KQIQKHKKIA
 c09g51   .......... .......... .......... .......... .........E
t20b129   .......... .......... .......... .......... ..........
 t24d81   .......... .......... .......... .......... ..........


          701                                                       750
 t21f21   ....GEKIDE ISAKLFPLLF TAFNIFYWFY YIGMSGGFF. ..........
f09c121   ....GARVDS FAAKAFPAMF AAFNG..... .......... ..........
 c53d63   ...TPERIDS ISSVMFPFSF FVFNIIYWFY YIHRKEIIKQ NLINRVDG..
 k10d61   ...SPEKIDR VSAIMFPACF AIFNIVYWSY YYNKKLEKAA EMKLNEDRL.
f55d105   ...TTEEIDR LSMIMFPGLF TLFNIIYWTY YLTVNT.... ..........
 f58g64   ...RPETVDF YSAIFFPTAY MLFNISYWSF YLTSLSEYFD EDVNIDQP..
 f47a41   .......... .......... .......... .......... ..........
 unc49c   .....SHIDI VSRAAFPLVF ILFNTLFWLI LL........ ..........
 unc49a   .....SNIDK YSRSLFPSIF VLFNVGYWAY FIRQSQIQEE QRNSQIL...
 f11h82   .NGAWGKVDL FSRFAFPIFF VIFHIVYWTY YINQ...... ..........
    hg4   ....PAKIDY YARFCVPLGF LAFNAIYWTS CLVMVSRLV. ..........
 gluclβ   ....PAKIDF YARFVVPLAF LAFNVIYWVS CLIMSANAST PESLV*....
 Cegbr3   ....SRRVDL MSRLTFPLTF FSFLIFYYVA YVKQSRD*.. ..........
 Cegbr2   ....SKRIDV VSRLVFPIGY ACFNVLYWAV YLM*...... ..........
 gluclα   ....SKRVDL ISRALFPVLF FVFNILYWSR FGQQNVLF*. ..........
 c27h58   SRGRAERIDI IARIIFPLAF ILFNFAYWSI YLEEEDPDES ..........
    hg1   ....VNRVDD VSKVIFPSLY VLFNILYWVA FLYWIPDEVD QIQGITDIKP
c39b102   .......... .......... .......... .......... ..........
 c45b24   GRNRAKKIDQ TSRWIFPLTF IIFNLTYWIY YLYWKE.... ..........
 c09g51   NRKNAQKIDR YSRALFPLAF IIFNIFYWIY YLKYAGSNSP ELLL......
t20b129   ...TSVQVDR CSMIMFPLSF LIFNVILVNI FHENGSSHVN SSHSLFPHAL
 t24d81   .......... .......... .......... .......... ..........
                     TM4
```

```
          751                                                 790
 t21f21   .......... .......... .......... ..........
f09c121   .......... .......... .......... ..........
 c53d63   .......... .......... .......... ..........
 k10d61   .......... .......... .......... ..........
f55d105   .......... .......... .......... ..........
 f58g64   .......... .......... .......... ..........
 f47a41   .......... .......... .......... ..........
 unc49c   .......... .......... .......... ..........
 unc49a   .......... .......... .......... ..........
 f11h82   .......... .......... .......... ..........
    hg4   .......... .......... .......... ..........
 gluclβ   .......... .......... .......... ..........
 Cegbr3   .......... .......... .......... ..........
 Cegbr2   .......... .......... .......... ..........
 gluclα   .......... .......... .......... ..........
 c27h58   .......... .......... .......... ..........
    hg1   *......... .......... .......... ..........
c39b102   .......... .......... .......... ..........
 c45b24   .......... .......... .......... ..........
 c09g51   .......... .......... .......... ..........
t20b129   PPLLVFVLFK NHRIFSYYFI TGMLKLAIKS PQKSWQWALR
 t24d81   .......... .......... .......... ..........
```

10.2 Table of Identities of nematode inhibitory TGICs

The sequence alignment above was used to calculate a table of amino acid differences using the GCG program DISTANCES (Devereux *et al.* 1987). To calculate the percentage identity, the figure given must be subtracted from 100.

Matrix Number	Accession number or subunit name
1	t21f21
2	f09c121
3	c53d63
4	k10d61
5	f55d105
6	f58g64
7	f47a41
8	*unc*49c
9	*unc*49a
10	f11h82
11	hg4
12	glucIβ
13	Cegbr3
14	Cegbr2
15	glucIα
16	c27h58
17	hg1
18	c39b102
19	c45b24
20	c09g51
21	t20b129
22	t24d81

Matrix 1: Part 1

	1	2	3	4	5	6	7	8	9	10	11	12
1	0.00	44.11	57.95	56.39	61.84	59.95	57.19	71.13	70.74	70.50	72.57	71.47
2		0.00	62.10	61.74	66.37	61.88	65.64	74.20	75.00	75.72	75.13	72.80
3			0.00	39.50	51.79	52.00	53.30	73.41	73.10	75.21	74.38	75.41
4				0.00	53.98	50.49	57.35	72.22	71.88	72.13	74.87	74.68
5					0.00	55.53	58.98	73.96	75.68	75.61	76.52	75.97
6						0.00	44.06	72.93	73.97	74.67	75.94	74.27
7							0.00	72.70	73.32	72.16	76.22	75.58
8								0.00	26.35	55.49	65.89	65.19
9									0.00	53.83	67.11	67.10
10										0.00	66.30	66.20
11											0.00	21.65
12												0.00

Matrix 1: Part 2

	13	14	15	16	17	18	19	20	21	22
1	70.85	70.75	70.26	71.70	74.52	73.87	71.19	71.63	62.11	72.45
2	73.42	73.48	73.68	77.65	78.08	76.88	75.27	74.79	62.75	72.58
3	72.70	72.52	72.59	77.51	79.30	76.27	72.41	71.76	64.78	72.24
4	71.28	71.28	74.16	78.41	79.56	75.62	70.62	68.77	64.89	69.40
5	75.71	74.40	76.36	80.45	79.03	76.31	78.04	75.77	68.61	76.36
6	72.01	71.75	72.77	74.34	76.28	74.54	74.81	72.58	68.04	73.57
7	74.49	74.17	74.32	79.95	77.09	73.74	73.07	72.90	65.75	72.15
8	66.57	66.08	66.76	68.62	68.61	74.93	74.35	72.89	71.55	74.64
9	65.60	65.56	66.92	70.48	71.13	73.44	74.05	74.74	74.47	73.39
10	66.10	66.57	68.21	70.41	69.39	74.36	72.80	73.96	73.64	74.33
11	56.25	53.48	55.13	63.57	72.61	71.14	72.30	73.24	74.74	70.53
12	55.23	52.64	56.52	63.38	73.40	71.39	71.03	72.12	73.11	69.77
13	0.00	17.68	45.41	61.79	69.03	69.08	69.44	68.39	72.99	66.89
14		0.00	42.75	59.02	69.21	69.64	69.32	68.82	72.78	67.47
15			0.00	65.87	73.46	68.68	69.94	70.68	74.24	70.96
16				0.00	73.61	76.17	72.09	73.27	76.15	76.36
17					0.00	74.49	73.16	77.14	77.70	73.97
18						0.00	65.97	65.85	77.86	67.17
19							0.00	54.30	76.26	49.70
20								0.00	75.69	55.39
21									0.00	74.32

11. Appendix 2. *Eschericia coli* genetic markers

Marker	Description
Amy	Amylase
ara	Mutation destroys ability to use arabinose
Cam^r	Chloramphenicol resistance
endA1	DNA specific endonuclease 1, mutation improves quality and quantity of miniprep
F	Contains the F plasmid
gyrA46	DNA gyrase subunit A
hsdR($r_K^- m_K^+$)	Ablates type I restriction but not methylation of *E. coli* strain K.
lac	Unable to utilise lactose
LacIq	Overproduces the *lac* repressor protein
LacZ	β-Galactosidase
lacZM15	Specific N-terminal deletion permitting α-complementation
ProAB	Requires proline for growth
recA1	Recombination deficient.
relA1	Permits RNA synthesis in absence of protein synthesis
rpsL	30S ribosomal subunit S12: mutation confers streptomycin resistance
SupE44	Suppressor of amber (UAG) mutations.
thi	Requires thiamin (vitamin B1) for growth
Tn10(tetR)	Contains the TN10 transposon, conferring tetracycline resistance